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THE EFFECTS OF EXTIRPATIONS ON HIGHER BRAIN PROCESSES

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Observation of behavioral adjustments after injury to the nervous system has been, for more than a century, one of the principal sources of knowledge concerning the functions of the brain. Refinements of the extirpation technique, introduced in recent years through the development of neurosurgery and by elaboration of the earlier methods of studying the behavior of animals, have made possible new experimental studies of the correlation of behavior and neural structure. With this development has come recognition of the need for knowledge of the behavior mechanisms of the normal subject in order to understand the nature and significance of defects associated with cortical injuries. Comparison of defects associated with occipital injury in rat, monkey, and man may be seriously misleading because of the different rôles played by vision in their normal action systems. The almost constant visual alertness in primates is in contrast to apparent lack of visual dominance in the rat. Studies of sensory control of the maze habit further indicate the lack of sensory dominance among the several sensory avenues. Lashley (1938, p. 180) has emphasized a further difference in the apparent dominance of remote over local visual cues and points out that "the conditions of visual attention must be quite different in the rat from those in man" (without detracting thereby from the many similarities of visual organization in rat and in man). Closely allied to the question of sensory dominance is the "encephalization of function"—the progressive shifting of function, in the phyletic series, from older to more recently developed nervous structures and by the increasing importance of the higher centers in the regulation of behavior (Dusser de Barenne, 1934; Fulton and Keller, 1932; Marquis, 1934). While the concepts of sensory dominance and encephalization of function are too general for application to a specific

instance, except as it may be experimentally established, attention to these principles may contribute to better evaluation of a particular experiment.

One of the outstanding changes in our views of the functions of the cerebral cortex during the past decade has been recognition of the close interdependence of the cortical and subcortical structures in the mediation of behavior. Not only have behavioral modifications, formerly thought to be characteristic of cortical function, been shown to occur at subcortical levels, but many presumably lower center instinctual functions have been found to be significantly influenced by cortical lesions. Because of this change in emphasis, studies relating to the subcortical mechanisms and learning at "simple" sensory-motor levels, as well as to behavior at the traditionally "higher" levels, have been considered in some detail.

Response to brightness differences. Knowledge of the neural correlates of optically induced behavior has increased greatly since Munk's localization of a visual center in the occipital lobes, and the formulation of experimental problems has changed from a rather futile attempt to determine whether a given bit of behavior involves "seeing" to the investigation of the neural and stimulus control of measurable visual capacities. It is clear from experiments with varying behavioral methods and on a variety of animal subjects that in the absence of the occipital areas certain responses to optical stimulation, other than the ocular reflexes, remain intact or, if disturbed by the lesion, can be reacquired.

Three series of experiments with essentially comparable methods have demonstrated that discrimination of differences in intensity of two stimulus patches is possible after complete bilateral destruction of the striate areas (in the rat, Lashley, 1935b; in the dog, Marquis, 1934; in the cat, Smith, 1937b). Using a conditioned lid response to an increase in intensity, Marquis and Hilgard (1936, 1937) have demonstrated the capacity of the dog and monkey to respond to changes in light intensity after removal of these areas. Poltyrew and Zeliony (1930) and Culler and Mettler (1934) have reported establishing simple conditioned responses to sound and light in decorticated dogs. Kluver (1936) has reported evidence of response to changes in intensity after removal of the occipital lobes in monkeys and was further able to show that response occurred to a sudden decrease in light as well as to an increase—the condition to which the animal had originally been trained. It has not thus far proven feasible to apply equally rigorous testing

techniques to the problem of response to brightness in man after bilateral occipital lobectomy, but Marquis (1934) after a review of clinical materials concluded that "destruction of the visual area of the cerebrum in man results in a complete loss of all visual sensations and of all optic reflexes with the exception of pupillary constriction" (p. 574). With possible exception of man and the anthropoid apes, which have not been examined, it is clear that animals lacking the visual cortex can form habits based on certain aspects of optical stimulation.

Two problems arise from these observations: 1, the characteristics of visually controlled behavior in animals lacking the striate areas, and 2, the other cortical or subcortical structures concerned in the mediation of these habits. Lashley's observations on the rat indicate that in addition to response to intensity differences, a sort of primitive detail vision—the ability to distinguish differences between two masses of light—is retained. Smith and Marquis have reported similar results for the cat and dog. In the monkey (Kluver, 1936) attempts to demonstrate response to position or direction of light masses have given no indication of retention of this ability after complete occipital lobectomy; either increase or decrease in intensity of the test objects or in the general illumination of the room were the only aspects of optical stimulation which evoked differential responses.

Threshold determinations for the discrimination of brightness differences between two lights of unequal intensity have been made in the rat (Lashley, 1930), in the dog (Marquis, 1934) and in the cat (Smith, 1937). Thresholds in the dog and cat, obtained under conditions of minimal illumination of the surrounds of the stimulus masses have shown a slight but not very significant increase after complete extirpation of the striate areas. Tests on the rat under diffuse daylight conditions indicated rather large increases in threshold values for the operated animals. A possible explanation for this difference is found in Smith's experiments on cats, which were tested under three conditions of the surrounds: minimal illumination, low general illumination and high general illumination. There was no marked variation in the ability of the normal animal to discriminate differences in intensity of the stimulus patches under conditions of low and high general illumination. On the other hand, extirpation of the striate areas permanently impaired ability to make discriminations under conditions of high general illumination. While the evidence clearly shows that responses to differential brightness cues can be established in the absence of the occipital areas and may under favorable circumstances appear to be not

inferior to those found in the normal animal, the loss of the geniculo-cortical system places certain limitations on the functional capacity of the remaining visual mechanisms.

While these limitations in the discrimination of brightness by the animal lacking the striate cortex have been emphasized, there are indications that in some respects the organization of visual activity related to brightness is not greatly different in the normal and operated animals. Hebb (1937 a, b; 1938) has presented evidence showing that the figure-ground relationship, the perception of identity in certain geometrical figures and the transfer of response in the discrimination of brightness and size are innately determined. In the normal animal discrimination of brightness appears to be relative rather than absolute. When subjects trained to select the more intense of two stimuli are confronted with hitherto unexperienced intensities, they react on a relative rather than an absolute basis even though in doing so it may require rejection of the previously positive stimulus. This characteristic of brightness discrimination in not significantly altered in animals in which the geniculo-striate system has been damaged or completely destroyed. Capacity for relational response is not restricted to cortical tissue.

In still another respect the brightness discrimination habit in the normal and operated subjects may be indistinguishable. Rate of acquisition of the habit in subjects lacking the striate area is not significantly different from normal animals as measured by trials and errors made in learning. (Marquis' observations on the dog suggest post-operative retardation but probably not of significant magnitude. See also Ghiselli, 1938.) Similarly acquisition of the conditioned lid response to changes in intensity of illumination, except for a slight increase in the latency of response, was not measurably different in normal and operated dogs and monkeys.

This apparent normality of acquisition in the absence of the striate areas, when considered in relation to postoperative amnesia after destruction of this region in animals that learned the brightness habit before operation, presents a perplexing problem which has not as yet found a satisfactory explanation. Tests of postoperative retention of the reaction to light in the Yerkes box (Lashley, 1935b) have been consistent in showing complete loss of the habit after destruction of the striate cortex, a finding that has also been confirmed in the cat and dog (Smith and Marquis). The habit is somehow dependent on the striate cortex when learned by the intact subject yet in the absence of

this region it can be acquired within normal limits of training (cf. Ghiselli, 1938). While retention of the habit is in some manner dependent on the striate cortex Lashley's evidence shows that it is not dependent on any particular part of this area. As long as any part of geniculo-striate system, even a few cells connected with the nasal retina, remains intact, the brightness habit may be retained without loss.

Results with the conditioned lid response to changes in intensity have given somewhat different results than those obtained with the Yerkes discrimination box. Marquis and Hilgard (1936) found perfect retention after destruction of the occipital lobes in the dog. They conclude: "Two lines of evidence support the view that the brain mechanism mediating the conditioned response is the same in normal dogs as after removal of the visual cortex: (1) the similarity of all characteristics of the conditioned response before and after operation, and (2) the retention without retraining of a conditioned response acquired prior to operation. The results indicate, not that vicarious functioning has taken place, but that the normal neural mechanism of conditioning has been practically undisturbed by removal of the occipital lobes. Evidence is presented that the essential locus of this neural mechanism is subcortical" (p. 177). Findings on the monkey, however, were equivocal in that two animals showed partial amnesia and one subject good retention of the habit (1937).

This difference in findings may be, in part at least, a function of the different demands on the organism made by the discrimination box and conditioning techniques. The discrimination box required response to the direction of stimulation (possibly a primitive form of pattern vision) as well as response to brightness whereas the conditioning method required response only to changes in intensity without regard to direction (cf. Kluver, 1936 and above). Also different motor mechanisms are involved since the discrimination box utilizes the greater part of the skeletal musculature while the conditioned eye-lid technique requires only the restricted musculature of the corneal reflex. The significance of these factors and the identification of the subcortical centers concerned in these responses remain to be determined.

Response to visual detail. A similar situation appears to prevail with respect to mechanisms of detail vision. While it is clear that for certain aspects of detail vision the intactness of the striate cortex is important, Smith (1938) has questioned the current emphasis on the striate cortex as the sole neural mechanism concerned. In support of

this position he considers his own observations (1937a, 1938) and those of Ter Braak (1936), which show that removal of the striate cortex in several infra-human mammals does not significantly alter the form of optic nystagmus evoked by complex patterns (alternating black and white bands). Similarly the optic nystagmus produced by apparent movement remains after loss of the occipital lobes. Tests for visual acuity, using the optokinetic responses as indicator, show no superiority of the geniculo-striate system over the subcortical mechanisms involved in these reactions. One important difference has been noted, however, in that the animal lacking the striate cortex fails to respond to a single line or to widely separated stripes. It is suggested that this loss is a specific defect in visual capacity associated with impairment of the central mechanisms for foveal vision. Smith and Bojar (1938, p. 211) conclude: "The view that the cortical centers of vision are the only neural centers sufficiently organized to mediate a highly refined visual acuity is refuted by the results and interpretations presented above."

While these forced reactions to moving visual patterns may partake of some of the characteristics commonly associated with pattern vision, it is clear that the geniculo-striate system is essential to the more varied adjustments involving response to visual detail and indeed to at least one aspect of optic nystagmic response. With the development of adequate techniques for examining detail vision in the rat, Lashley (1931) and Lashley and Frank (1934) have shown visual memory, as tested by differential response to inverted and upright triangles, is not conditional on any extra-striate area or on any part of the striate area other than the projection field of the temporal retina. While damage to this cortical field may result in disturbance of the habit, perfect retention can in some instances be obtained if a part of the superior or inferior temporal projection in one hemisphere remains intact. However, within the projection field of the temporal retina there is no evidence of localization of the habit. Evidence has also been presented that the visual cortex is essential to perception of distance as measured by force exerted in jumping various distances (Lashley, 1937). Spence and Fulton (1936) and Poljak and Hayashi (1936) have shown that in primates visual acuity suffers a slight impairment with complete extirpation of one striate area and failure to discriminate after destruction of the macular area and extensive damage to the peripheral projection areas of the second hemisphere. There was indication, however, after the second operation that the animal could respond to the position of the two test fields, to light and to movement of objects in parts of the visual

field. Kluver's observations (1936) indicating that after complete bilateral occipital lobectomy monkeys fail to utilize visual cues in orienting themselves in space or in manipulating their environment have already been discussed. His studies involving subtotal lesions of the occipital lobes (1937) demonstrate a striking retention of visual function. The test situations include measurement of responses to differences of brightness, color, form and size, responses to sound, weight discrimination and reaction times. When cases with unilateral lesions of the occipital lobes were examined postoperatively, it was found that differential responses to the above tests were either not lost at all or disturbed only slightly. In the few instances of loss, the preoperative level of performance was quickly reached. Postoperative thresholds were similar to those obtained before operation. No serious limitation of ability to "generalize," i.e., to detect similarities in heterogeneous situations, was observed.

Bilateral destruction of the macular regions resulted in apparent blindness for the first few days. By the fourth week the utilization of visual cues in reaching for and grasping food or other objects and in responding to people in the room was not markedly different, except for field defects, from the behavior of normal monkeys. During the first days of experimental testing the differential responses to visual stimuli were disturbed under some conditions. At the end of four weeks the differential responses established preoperatively were again intact. Threshold determinations involving color and form were not altered. On the other hand, performances were not entirely errorless and variations in the test situations disturbed the subject to a greater degree than before operation. The surprising feature of the experiments is the degree to which visually directed behavior resembled that of normal monkeys.

Responses to auditory stimuli. The experimental studies of learning based on other sense modalities are less complete than those in the field of vision. The earlier observations of Munk and of Rothmann, that complete bilateral destruction of the auditory areas is essential to induce serious impairment, have been confirmed by several investigators. The experiments of Wiley (1932, 1937) and Pennington (1937, 1938) reveal a marked similarity between the acquisition of simple auditory discrimination habits and the learning of the brightness habit in rats. Wiley has shown that initial postoperative acquisition of a differential response to noise is not significantly inferior to the rate of learning by a normal subject. On the other hand, postoperative retention is seri-

ously impaired by such lesions. Pennington similarly found that a learned differential response involving localization of the sound source was lost after extensive injury to the lateral surface of the brain. Initial postoperative acquisition of the response was not affected by lesions of the auditory areas. Extensive lesions in other areas of the cortex did not influence learning or retention. The critical cortical area, as determined by these behavioral techniques, was found to coincide with the cortical projection field of the medial geniculate nucleus. There was no evidence of subordinate specialization within this region. Odani (1935), failing to find localization of a similar response to noise, concluded that retention of the habit was due to the primary activity of the subcortical centers. The more carefully controlled experiments of Wiley and Pennington seem conclusive, however, in indicating the participation of the cortex in the intact organism.

The experiments of Girden, Mettler, Finch and Culler (1936) on retention and acquisition of conditioned responses to acoustic, tactile and thermal stimulation after complete decortication are of particular significance. In the normal dog or in one with extensive cortical injury, the first form of conditioning involves a diffuse response and it is only later that the localized adaptive response (withdrawal of paw) develops. In the fully decorticate dog this diffuse conditioning develops as rapidly as in the normal but the localized adaptive response cannot be obtained in the decorticate specimen. Development of this highly differentiated response appears to depend on the presence of cortical structures. (Conditioned responses were established to acoustic, thermal and tactile stimuli.) A differential response to acoustic stimuli, with maintenance of a vigorous response to a bell during gradual decline in response to a 1000 $\sim$ tone, and extinction and reestablishment of the conditioned response to a loud tone, were demonstrated.

Sensory discrimination. The progressive corticalization of sensory function in primates, as measured by tests for discrimination of weight and surface texture, has been investigated by Ruch and Fulton (1935) and Ruch, Fulton and German (1938). In the monkey, no disturbance of weight discrimination ability was observed after lesions of the postcentral gyrus and the posterior parietal lobules. Lesions involving both of these regions resulted in some permanent deficit although not complete abolition of the ability. Similar tests with the chimpanzee indicate some initial postoperative impairment which disappeared with retraining. In clinical cases, studied with similar methods for thresh-

old determinations, the initial disturbance of function was greatly ameliorated through specific training although a residual deficit remained. The clinical and experimental findings are in agreement in indicating that the capacities concerned are not focally represented in the parietal lobe and that after subtotal lesions a surprising degree of recovery can be brought about through specific reëducation. The structures involved in this recovery, whether the motor areas, remaining portions of the parietal lobe, or the thalamus, are not at present known.

Olfactory discrimination. The mechanisms involved in learning and retention of olfactory discrimination by the rat differ considerably from those of the other sense modalities in that the cerebral cortex is not directly concerned. Swann's (1934, 1935) striking results indicate that destruction of various parts of the archipallium, including the hippocampal complex, pyriform lobes, septum, and amygdaloid nucleus, and interruption of the main fibre tracts to and from the archipallium—although not complete in any one animal—resulted in no interruption of olfactory discrimination. Dependence on particular structures could be demonstrated for only the anterior limb of the anterior commissure and the olfactory bulbs. Brown and Ghiselli (1938) have shown that unless all incoming fibres were lost, olfactory discrimination could be established. Ablation of the olfactory bulbs resulted in complete loss of the habit, which demonstrates the adequacy of the technique for detecting losses of olfactory discrimination. Destruction of as much as 85 per cent of the cerebral cortex revealed no amnesia for the olfactory discrimination habit although it did produce profound sensory and motor disturbances and aimless running in the test apparatus. This period of aimless activity was rather suddenly replaced by motivated behavior, and once this change occurred, the olfactory discrimination was executed without evidence of loss. While the identification of the subcortical structures involved in this habit has not been made, the results do indicate that the cortex is not directly concerned in acquisition and retention of this olfactory discrimination. Comparable studies are not available for other animals.

The functions thus far considered have related primarily to a single sensory modality and have shown, e.g., in the rat, quite different characteristics of cerebral organization. In various instances there is dependence on a small localized area of the cortex involving spatial distribution of elements (pattern discrimination), dependence on a particular cortical area in the intact subject although the habit can be mediated through other mechanisms in the absence of the cortex (brightness

discrimination and sound localization) or, on the other hand, independence of any cortical area, even for a general facilitating background (olfactory discrimination).

Within the level of complexity for the problems involved, there was no indication of involvement of association areas apart from the primary projection areas. Problems demanding abstract visual generalizations have not been investigated although preliminary studies by Kirk (1936) with more complex patterns suggest that the discrimination habit may involve some extra-striate functions. On the other hand, a considerable body of data indicates that in the rat the primary projection areas subserve not only their immediate sensory functions but also participate in the integrating activities of the cortex in a manner that is independent of the other sensory functions. This latter type of integration shows little functional localization in the cerebral cortex, and a considerable relationship between efficiency and the mass of available cortex.

Maze learning in rats. The comprehensive studies by Lashley (1929) and Lashley and Wiley (1933) have demonstrated a consistent impairment of maze learning ability following damage to the cerebral cortex.

The relationship between extent of injury and degree of deterioration was found to be curvilinear, error scores being a logarithmic function of the size of the lesion. Locus of the lesion, on the other hand, bore little relationship to retardation. Cortical destruction within the fronto-motor, somesthetic and visual areas caused approximately equal degrees of deterioration, whereas lesions in the auditory region, although they resulted in impairment, were slightly less damaging. Relative difficulty of simple and complex mazes (obtained by duplication of identical elements) was found to be the same for operated and control animals. While the authors do not regard the results as conclusive for the exact equivalence of the several areas, a considerable degree of equipotentiality of the various regions of the cortex in mediating the maze habits is indicated. In considering the above data and other studies, the authors conclude that "the uniform trend of the data . . . suggests that there must be some constant causal factor in consistency of maze performance dependent upon the mere quantity of cerebral tissue and not an artifact arising from the limitation of this or that special function" (p. 47). These results and interpretations, considered in relation to the specific sensory functions known to be mediated through the various sensory spheres, attribute to a given sensory or motor region participation in two somewhat disparate types of functions. One is the mediation of habits and functions related to the

sensory modality in question; the other is of a more general nature and is concerned with integrations above a simple sensory-motor level.

Behavioral analyses of maze performances implicate not merely one but several sensory modalities in the stimulus control of this habit and deprivation through peripheral loss of the receptor may seriously disturb performance of the maze habit. Hunter (1930, 1931; see also Lashley, 1931) while not denying other than sensory functions to a projection area, has pointed out the possibility that the deterioration in the maze performance may be due primarily to sensory deprivation—the larger lesions resulting in disturbances in several sense fields—rather than to impairment of a non-localized integrating capacity.

Tsang's experiments (1934, 1936) were designed to test this question as it relates to the most highly developed sensory projection system in the rat, i.e., the visual. If the functions of the striate areas are purely sensory, then peripheral sense deprivation and destruction of the striate areas should evoke equally severe deterioration. Such was not the case. Cortical destruction, although some visual functions could still be mediated through subcortical paths, resulted in significantly greater deterioration than enucleation of the eyes. Furthermore, the impairment after cortical injury does not seem to be due to disruption of an acquired intra- or inter-sensory organization of space habits. In Tsang's second experiments the animals were peripherally blinded before the eyes were opened and presumably deprived of any visual functioning of the striate area or of sub-cortical mechanisms. Animals thus blinded in infancy were not significantly inferior to the normal controls in acquisition and retention of the maze habit. Subsequent destruction of the striate areas caused marked retardation and the animals exhibited the behavioral characteristics of normal animals which had undergone similar cortical injury. These experiments do not exclude or deny the possibility of deterioration in maze performance being due in part to sense deprivation from cortical ablation. They do suggest, however, that the striate areas participate in non-visual integrative functions of the cerebrum although the mechanisms of such participation are not now known.

Other complex adaptations in rats. Evidence of a non-localized integrating function has been reported by other investigators on various types of problems. The delayed alternation problem (Loucks, 1931) required that the animal alternately choose the right or left pathway in a maze and imposed the further task of remembering the correct

path during a fifteen second interval between trials. Extirpation of the cortex in the fronto-motor region prior to training resulted in severe impairment in the rate at which the problem was learned and in the case of subjects operated after training, in severe amnesias which were proportional to the magnitude of the injury. Maier's experiments (1932, 1938) involved two types of behavioral organization, one involving combination of isolated experiences (reasoning) and the other association of contiguous experience (learning). The former type appears to be more susceptible to cortical injury than the latter and suggests the independence of neural mechanisms mediating the two types of behavioral organization. As in the maze experiments cited above, deterioration of "reasoning" ability was not exclusively associated with injury to a specific cortical region but followed lesions in any part of the dorsal convexity of the cerebrum. The severity of impairment was related to magnitude of cortical defect as expressed by the correlation coefficients of 0.54 and 0.75 for the anterior and posterior regions of the cerebral cortex.

Problems calling for manipulation of specific features of the environment, such as depression of a bar or tearing through a strip of paper, yield similar evidence. Retardation in rate of learning follows injury to various cortical areas. While some of this retardation can be attributed to sensory defects, the greater retardation associated with cortical injury rather than with mere sense deprivation indicates that other factors—restriction in variety of exploratory acts, in time spent in exploration and in development of manipulative patterns adapted to the specific latches—are probably operating. These latter deficiencies are characteristic of animals with extensive brain lesions without respect to the locus of the injury. Other latch box problems capable of solution without the development of specific adaptive movements are learned in normal time after extensive lesions of the cortex. Lashley (1935a) has interpreted these data as indicating "that the mechanism of association, as such, is not disturbed by cerebral lesions and that retardation from cerebral lesions is due rather to disturbance of such functions as are implied by the terms attention, insight and initiative" (p. 38).

Stereotypy of behavior after cortical injuries has been noted by various investigators (Hamilton and Ellis, 1933; Lashley, 1929; Maier, 1932). Experiments on variability and plasticity in behavior indicate similar limitations in the behavior of cortically impaired animals. The behavior of an intact animal when placed in a novel situation is charac-

terized by a series of attempted solutions directed toward various aspects of the environment. In the normal subject a given mode of behavior is persisted in for a time, then dropped and replaced by a new approach, which in turn may again be supplanted by still another attempted solution. The effect of even small cortical injury is to restrict severely the number and complexity of attempted solutions. The nature of this restriction seems in part to be a function of the sensory areas involved rather than total extent of cortical injury (Kreshevsky, 1935). In other test types the subject was confronted by a maze which allowed different but equally adequate solutions. It was found that the cortically impaired animal was not only inferior in arriving at a simple generalized solution to the problem, but it had investigated *fewer* possible solutions than the normal subject. This defect bore no relation to the locus of the lesion although severity of defect correlated with the magnitude of the injury (1937). Similar defects were observed in another problem which allowed two solutions: the one, a short path to the food which necessitated the animal's losing sight of the food for a short time; the other, a much longer path but one on which the food remained within the visual field. The normal rat was capable of adopting the former type of solution when it resulted in more efficient performance and the tendency to use this solution increased with practice. On the other hand, rats which had suffered small cortical lesions exhibited marked preference for the longer route which kept the goal in view both in the early trials and after considerable training in this problem. This decrement in adoption of "umweg" type of solution followed small cortical lesions and was not related to either locus or magnitude of cortical damage.

Studies of innate maternal behavior patterns in primiparous rats (Beach, 1937, 1938) indicate that the cortex contributes an integrating and directing component to this behavior. Many of the defects in behavior do not point to a specific sensory or motor deficit but to an inability to direct behavior toward a definite end. Nest building in the cortically operated animal begins later in the pre-partum period than does that of the normal or peripherally blinded subject and the quality of the nest, when built, is inferior. The partially decorticated mother may spend as much time as the normal animal in carrying its pups around the cage but at the end of the period the litter of the operated animal is still scattered about the cage while that of the normal mother has been collected in a nest. As in one of the maze situations, peripheral deprivation of one or another of the sensory avenues

resulted in much less disturbance of this instinctive behavior pattern than did damage to cortical tissue. No particular portion of the cortex appears more important than any other part and the severity of deficit bears some relation to extent rather than locus of the lesion.

The failure to find evidences of functional localization of more complex adaptations has been a consistent result in studies of cortical function in the rat. In these activities the cortex appears to function more as a dynamic whole than as a highly specialized structure although the theories of mass action and equipotentiality have been criticized as violating principles of structural organization of the nervous system (Mettler, 1935).

No conclusive evidence has been presented in support of the so-called association areas in the rat. This lack of evidence may reflect inadequacies of behavioral techniques and the difficulties of producing, in a brain as small as that of the rat, lesions of a discrete area without at the same time interrupting fibre tracts to areas beyond the confines of the primary cortical destruction. The anatomical studies of the primary projection fields in the rat cortex indicate adjacent regions which may well be associational centers that have thus far escaped detection.

Higher levels of organization in primates. The experimental and clinical studies of the primates leave little doubt concerning the existence of specialized fields for the elaboration of more complex aspects of behavior. The temporo-parietal and frontal regions appear to be of special significance, and injury to these regions results in particular kinds of behavioral impairment. Within the more strictly motor portions of the frontal lobes, lesions of the premotor areas have been observed to disturb the ordering of a series of acts although it leaves the execution of isolated movements relatively intact (Jacobsen, 1934). Richter and Hines (1938) indicate that injury to a different region, Brodmann's area 9, is accompanied by increased spontaneous activity.

The chimpanzee or monkey which has suffered extensive injury to the portions of the frontal lobe anterior to those just discussed shows other changes in behavior (Jacobsen, Wolfe and Jackson, 1935; Jacobsen and Nissen, 1937). The normal chimpanzee has considerable facility in using sticks or other objects to manipulate its environment, e.g., to reach a piece of food beyond its unaided reach. It can solve such problems when it must utilize several sticks, some of which may not be immediately available in the visual field. After ablation of the pre-frontal areas, the chimpanzee continues to use sticks as tools but it may have

difficulty in solving the problem if the necessary sticks and the food are not simultaneously present in the visual field. It exhibits also a characteristic "memory" defect. Given an opportunity to observe a piece of food being concealed under one of two similar cups, it fails to recall after a few seconds under which cup the lure has been hidden and it is as likely to select the cup containing no food as the correct cup (delayed response test). Capacity to perform the delayed response test has been shown in monkeys to depend on the intactness of a small portion of the frontal lobes. Extensive injuries to other parts of the cortex, the motor and premotor areas, parietal and temporal lobes, postcentral gyrus and incomplete lesions of the occipital lobes, caused no impairment of this capacity although such lesions might be accompanied by other disturbances (Jacobsen, Elder and Haslerud, 1936; Breslaw, Barrera and Warden, 1934; Settlage, 1937).

The deterioration of behavior is not by any means generalized but it is specific to certain situations. Many aspects of the animal's behavior are quite normal. Learning and retention of visual-kinesthetic-motor habits and of sensory discriminations are not impaired. Delayed conditioned reflexes, involving inhibition of response for ten to twenty seconds, may suffer some disturbance from frontal injury, but they are reëstablished without significant deviation from preoperative learning (Finan, 1937).

The observations of Kluver and Bucy (1938) on the effects of temporal lobectomy in the monkey present a type of disorder characteristically different from those observed after frontal, parietal or occipital lobe lesions. Motor reactions involved in walking, running, climbing and grasping were not impaired although certain other reactions were performed more slowly. Appreciation of size, shape, brightness and position were not significantly different from the normal monkey. There was but a slight visual field defect. Cutaneous sensitivity and motor reactions to various sounds were not impaired. Although there was no gross deficit in ability to discriminate visually, the ability to recognize and detect the meaning of objects by optical cues alone seemed to have been completely lost. The hungry animal picked up all objects within reach and after examination by mouth, discarded the inedible items. The same inedible object might be repeatedly picked up and discarded. Forms used in a discrimination problem apparently did not signify food but they were responded to as objects to be examined. Response to the figures did not appear to be a means to an end but an end in itself. Emotional responses were ap-

parently lost. Objects or actions which arouse fear, anger or resentment in the normal animal were accepted without sign of reaction. Although final evaluation of these results must await further anatomical study, it is apparent that they represent a type of defect not found after injury to the other cortical regions.

The fractionings of behavior observed after various injuries to the nervous system appear to have little relation to current psychological categories. Few, if indeed any, psychological functions that are derived from analysis of the activity of the whole organism are localized in a particular region of the brain. Thus, discrimination and response to relations are not peculiar to the cerebral cortex but they may also characterize the activity of subcortical centers. The defects associated with injury to the frontal lobe are not adequately encompassed by such terms as immediate memory, synthesis, temporal organization, or abstraction although these psychological functions may be modified by damage to this region of the brain. Similarly it is not of importance whether the defects described by Kluver and Bucy are classified as visual or agnostic for understanding their neurological mechanisms. At the present state of our knowledge of the inter-dependence of the so-called "elementary" and "higher" brain processes, description in experimental terms of the behavioral defects is more appropriate than elaborate psychological formulations.

SUMMARY

The cerebral cortex has long been regarded as the mechanism *par excellence* for the modification and integration of behavior, and some of the experiments here reviewed have added to our knowledge of these cortical functions. Equally significant, however, is the emphasis which must be placed on the subcortical centers in mediation of learned behavior. In the rat, learning based on some sensory modalities may be independent of the cortex, in other instances habits that are mediated through the cortex in the intact subject can be acquired with equal facility in the absence of the cortex, or, as in pattern vision, they may be dependent on the fine spatial distribution of cortical elements. Further, behavior mediated as subcortical levels is not necessarily of a primitive sort since it exhibits in some instances the same characteristics of response as cortically organized activity. Certainly the Pavlovian view which attributes modifiability exclusively to the cerebral cortex can no longer be maintained. In the higher mammals, and especially in the primates, the cortical centers have assumed a greater rôle than

in the rat, but even here it is clear that, with the possible exception of man, modification is not restricted to cortical mechanisms. The neural structures concerned in these subcortical activities and the manner in which the cortex and subcortical centers are organized remains largely unknown at present.

Development of specialized neural structures mediating the more complicated modes of behavior in the higher mammals seems to be clearly established. In the rat there is little evidence for specialized regions of elaboration other than the sensory and motor spheres, although this lack of demonstration may be due to inadequacies of method. On the other hand a number of studies have consistently indicated that the sensory and motor regions participate in a more general type of integration that is dissociated from their functions as sensory regions *per se*. Within limits all parts of the cortex appear to be equally capable of mediating this type of integration. In the primates, however, at least some of the more complicated behavioral adjustments clearly depend on the intactness of restricted areas, especially the parieto-temporal and frontal regions, and injury to these areas results in disturbances not found with damage to any of the sensory or motor projection fields.

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THE DETOXICATION OF CARBOCYCLIC COMPOUNDS

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The use of the term "detoxication" in relation to the chemical changes undergone by a compound in the animal body implies that such changes bring about an abolition or diminution of toxic properties possessed by that compound. A survey of the literature on "detoxication" reveals that chemical reactions occurring in the organism are frequently classed under this heading without reference to the toxicity of the compounds involved. It has become customary to describe as "detoxication reactions" those chemical changes occurring in the body which bring about the conversion of foreign compounds of known structure to derivatives which are excreted in the urine. It is in this commonly accepted sense that the term has been used in this review. It is necessary to recognize, however, that when used in this manner the term may be a misnomer in certain instances, for it does not necessarily follow that all foreign compounds are intrinsically toxic.

The term may be misleading in another sense. In the rat, benzoic acid is largely combined with glycine. When sodium benzoate is added to the diet of young rats, growth and survival occur only if the diet provides a supply of glycine which is sufficient for both the detoxication of the benzoic acid and the building of tissue proteins (85). In a similar manner there is inhibition of growth when bromobenzene is administered to young rats on a diet which normally contains just sufficient cystine and methionine for good growth. Growth is restored when the diet is supplemented with suitable amounts of cystine or methionine (278). It is known that bromobenzene can be converted by the rat to p-bromophenylmercapturic acid (N-acetyl-S-p-bromophenyl-cysteine) and the most probable explanation of the above results is that, under the experimental conditions used, the synthesis of mercapturic acid prevents growth by creating a deficiency of sulphur-containing amino acids. Experiments such as these indicate that in some cases the conjugation of foreign compounds takes precedence over essential physiological processes. This suggests the possibility that the toxicity of certain foreign compounds may be due partly to those

very reactions which lead to the formation of so-called "detoxication products."

Different opinions have been expressed concerning the type of mechanism which is responsible for detoxication. Sherwin (235) states: "It has been necessary for the body to call to its aid a chemical defense mechanism to guard against the poisons absorbed from the gastrointestinal tract. After many generations this chemical defense mechanism has been so perfected in its battle against putrefaction products absorbed from the intestine that it is now quite able to cope with many of the foreign organic compounds." An alternative point of view has been put forward by Quick (203) who, in considering the detoxication of benzoic acid, says: ". . . the idea is no longer tenable that these conjugations are more or less unimportant mechanisms concerned solely with the detoxication powers of the organism. If these synthetic processes are looked upon as normal and common chemical reactions made manifest because the body is applying them to a foreign substance, it is possible to perceive how a study of the conjugation of benzoic acid may help to solve various problems of metabolism which at present seems quite unrelated to hippuric acid and glycuronic acid." It should be pointed out that certain classes of compound which undergo detoxication are not known to occur among those formed during intestinal putrefaction, and also that a number of the changes to which foreign compounds are subjected in the organism are analogous to changes which occur in the course of normal metabolism. In the present state of knowledge, however, it is not possible to decide what type of mechanism is concerned in detoxication, and it may be that the types postulated by Sherwin and by Quick are both involved.

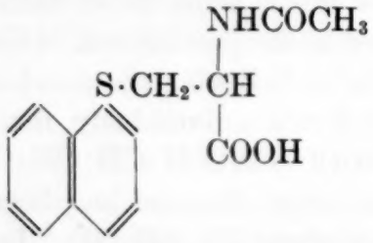
DETOXICATION REACTIONS. The chemical changes which some of the simple foreign carbocyclic compounds undergo in the animal body are reviewed below. For further information on the subject of detoxication, the excellent reviews written by Heffter (99), Sherwin (234), Ambrose and Sherwin (8), Harrow and Sherwin (98), and Quick (215) should be consulted.

Hydrocarbons. The fact that certain polycyclic hydrocarbons possess carcinogenic properties lends considerable interest to questions concerning the fate of hydrocarbons in the animal body. It has long been known that this class of compound undergoes oxidation in the body, for as far back as 1867 Schultzen and Naunyn (232) demonstrated that benzene can be converted to phenol by man and the dog. The oxidation does not stop at this stage, for catechol and quinol are also

formed (179). Other hydrocarbons undergo similar changes, and in consequence of this, questions concerning the detoxication of hydroxy-compounds are involved to a considerable extent in the study of the fate of cyclic hydrocarbons in the organism. That some of the phenol formed from benzene is excreted in a combined form was recognized by Munk (171), and it was later shown that this phenol is conjugated partly with sulphuric acid (16) and partly with glucuronic acid (143, 144). The tendency of phenolic compounds to combine with sulphuric acid disturbs the normal ratio of inorganic to organic (or ethereal) sulphates in the urine. This change has been shown to serve as an early sign of benzene poisoning in animals (285), and there is evidence that sulphate determinations on the urine are of value in the medical supervision of workers exposed to the possible risk of benzene poisoning (130). There are some grounds for believing that oxidative fission of the benzene ring can also occur in the animal body, for Jaffé (126) was able to isolate muconic acid, HOOC·CH:CH·CH:CH·COOH, from the urine of dogs and rabbits to which benzene had been administered. This was confirmed by later workers (78, 263, 59). In all cases the amounts of muconic acid obtained have been small. Jaffé considered that in the body larger amounts of benzene undergo ring fission than are indicated by the quantities of muconic acid which are excreted, for on injecting muconic acid he was able to recover only about 1 per cent of it from the urine. Similar experiments by other workers (164, 184, 59), however, have shown that a large proportion of an injected dose of muconic acid is excreted unchanged. Although benzene might be expected to give cis-cis muconic acid on oxidation in the organism (25), the form isolated is the trans-trans isomer (59). The question arises as to whether muconic acid is formed directly from benzene, or as a result of a metabolic disturbance produced by the hydrocarbon. Drummond and Finar (59) showed that both the trans-trans form of muconic acid and the cis-cis isomer are largely excreted unchanged after injection into rabbits. They observed, however, that the cis-cis isomer is partially converted to the trans-trans form when it is added to the urine of rabbits receiving benzene, although no such change occurs when it is added to normal urine. When the cis-cis acid is administered to a rabbit receiving benzene, only the trans-trans isomer can be isolated from the urine. Although these findings await explanation, they suggest that if cis-cis muconic acid is formed in the metabolism of benzene it may undergo conversion to the trans-trans form.

The metabolism of naphthalene is of especial interest in view of the

fact that when it is administered to rabbits in repeated doses a degeneration of the crystalline lens of the eye takes place which resembles that observed in human senile cataract (27, 5). Although qualitative tests for the presence of naphthols in the urine excreted after administering naphthalene have yielded conflicting results in the hands of different workers (117, 61, 191, 5), some oxidation of naphthalene to naphthol does occur, for Lesnik (146) was able to isolate α-naphtholglucuronic acid from dog urine after feeding naphthalene. In 1933 Bourne and Young (29, 30), after administering naphthalene to rabbits, isolated from the urine a compound to which, on the basis of their studies of its constitution, they assigned the structure:

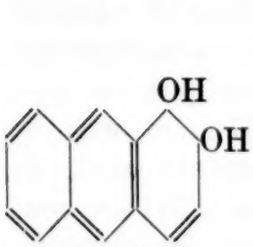


l-α-naphthylmercapturic acid

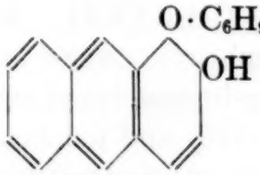
It was confirmed by synthesis (120) that the compound excreted is l-α-naphthylmercapturic acid. Its isolation removed the doubts which until then existed concerning whether or not hydrocarbons share with certain halogenated benzene compounds the property of being converted to mercapturic acids in the animal body. It has subsequently been shown by Stekol (247, 251) that other animals can synthesize l-α-naphthylmercapturic acid from naphthalene. It has recently been found that anthracene also gives rise to a mercapturic acid in the organisms of the rabbit and the rat (33) and the increased neutral sulphur excretion which follows the administration of benzene (37, 249) and phenanthrene (249) to the dog suggests that these hydrocarbons also yield derivatives of the same type. Baumann and Herter (16) obtained naphthalene by the steam distillation of the acidified urine of dogs to which naphthalene had been fed, and Bourne and Young (28, 30) observed that naphthalene is liberated from a soluble precursor by acidification of the urine of rabbits receiving naphthalene. Similar observations have been made with other animals (254). Although, in the rabbit, the compound which yields naphthalene on acidification is one of the main detoxication products of this hydrocarbon (28), all attempts to isolate it have so far failed.

We owe our knowledge of the metabolism of anthracene to the recent

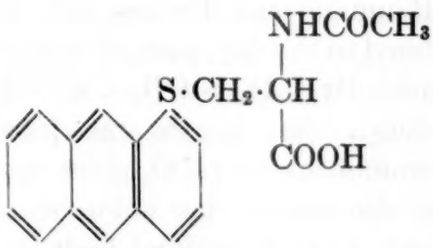
work of Boyland and Levi (31, 32, 33). They showed that the rat and the rabbit on a diet containing anthracene excrete 1,2-dihydroxy-1, 2-dihydroanthracene. Whereas the urine of the rabbit contains the d-form of this compound, the rat excretes the l-form (31). The rabbit also excretes d-1,2-dihydroxy-1,2-dihydroanthracene-1-glucuronic acid, and it is probable that the rat excretes the analogous laevorotatory compound (32). Some anthracene is converted to 1-anthrylmercapturic acid by both the rat and the rabbit (33). In addition to these compounds, the urine of the rat and the rabbit, on a diet which includes anthracene, contains a compound not yet isolated, which yields anthracene on acidification (33).



1,2-Dihydroxy-1,2-dihydroanthracene



1,2-Dihydroxy-1,2-dihydroanthracene-1-glucuronic acid



1-anthrylmercapturic acid

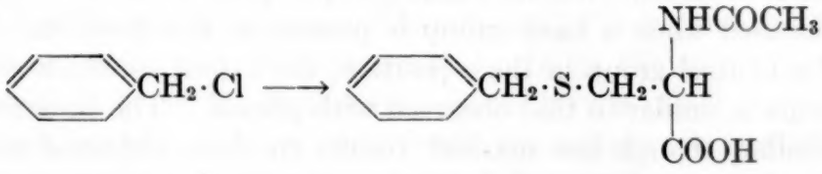
Evidence has been put forward by Bergel and Pschorr (22) that when phenanthrene is administered to the rabbit, phenanthrolglucuronic acid is excreted.

When either toluene (232, 138) or ethylbenzene (232, 262) is ingested it is oxidized to some extent to benzoic acid. There is no evidence of mercapturic acid formation after feeding toluene (37). Part of the dose of ethylbenzene is converted to phenylmethylcarbinol, $C_6H_5 \cdot CH(OH) \cdot CH_3$, and part to mandelic acid, $C_6H_5 \cdot CH(OH) \cdot COOH$. The former derivative is excreted in conjugation with glucuronic acid (262). Although n-propylbenzene, $C_6H_5 \cdot CH_2 \cdot CH_2 \cdot CH_3$, gives rise to benzoic acid in the body, this does not appear to occur in the case of isopropylbenzene, $C_6H_5 \cdot CH(CH_3)_2$, in which the side chain is branched. There is evidence that this compound is converted to a phenolic derivative (179). When two methyl groups are substituted in the benzene nucleus, as in m-xylene, $C_6H_4(CH_3)_2$, one is oxidized to a carboxylic group (80). In a similar manner, one methyl group of symmetrical trimethylbenzene (mesitylene), $C_6H_3(CH_3)_3$, is oxidized (176), as also is the methyl group in position 4 of 1:2:4 trimethylbenzene (pseudo-

cumene) (121). The changes which alkyl substituted benzene derivatives undergo in the body do not appear to be confined to the oxidation of substituent groups. For example, it has been reported (52) that mesitylene is not only oxidized to mesitylenic acid, but is also excreted partly as mesitol, $(CH_3)_3C_6H_2 \cdot OH$, and partly as p-hydroxymesitylenic acid, $(CH_3)_2C_6H_2(OH)(COOH)$. When triphenylmethane, $(C_6H_5)_3CH$, is fed to the rabbit and the dog, part of it can be recovered unchanged from the urine (163).

Halogenated hydrocarbons. The detoxication of certain halogenated benzene compounds has attracted much attention because of the effect of these substances on sulphur metabolism. It was first established by Baumann and Preusse (17, 19) that when bromobenzene is administered to the dog, part of it is transformed to p-bromophenylmercapturic acid, $Br \cdot C_6H_4 \cdot S \cdot CH_2 \cdot CH(NH \cdot CO \cdot CH_3)COOH$. Recently it has been shown that in the rat p-bromophenyl-cysteine (257), as well as bromobenzene (278), gives rise to p-bromophenylmercapturic acid. It is also known that chlorobenzene (125) and iodobenzene (21) are converted in the animal body to p-chloro- and p-iodo-phenylmercapturic acid respectively. The corresponding fluorbenzene derivative has not been isolated, but from the work of Coombs (47) it seems probable that it is formed when fluorbenzene is fed to the dog. According to Baumann (15) p-bromophenylmercapturic acid is present in the urine as an unstable complex with glucuronic acid which is easily split by dilute acids and alkalies. This derivative has not been isolated. Some bromobenzene is oxidized in the body to p-bromophenol and it is probable that o-bromophenol is also formed (19). Chlorobenzene gives rise to similar oxidation products (101). The phenolic derivatives so formed are excreted partly as ethereal sulphates and partly in conjugation with glucuronic acid. The fact that the mono-halogen substituted benzenes yield the corresponding p-substituted phenols in the organism has led certain workers (216, 240) to assume that this is a preliminary step in the synthesis of mercapturic acids. Experiments in which p-chlorophenol (48) and p-bromophenol (279) have been fed to animals have failed to reveal evidence of mercapturic acid formation. Results of this type, however, can only be regarded as indicative, for it does not follow that the changes which are undergone by a compound produced in the tissues are invariably the same as those undergone by that compound when it is administered to the animal. The negative results described above do not justify, therefore, a denial (48) of the possibility that phenol formation is a stage in the production of mercapturic acids in the or-

ganism. Some doubt exists concerning the detoxication of compounds of the dichlorobenzene type. No mercapturic acids have been isolated after administering these compounds to animals, although Baumann (15) obtained evidence of mercapturic acid formation after feeding o-dichlorobenzene. In the case of m- and p-dichlorobenzene he observed an increased excretion of ethereal sulphates, but could detect no mercapturic acid in the urine. The possibility that o- and m-dichlorobenzene may be converted to mercapturic acids in the dog is suggested by the observation of Callow and Hele (37) that these compounds have an effect on sulphur metabolism which is similar to that found in the case of monochlorobenzene. The nuclear substituted halogen derivatives of toluene tend to be oxidized in the body to the corresponding carboxylic acids (109) and no evidence of mercapturic acid formation has been found (15, 37). On the other hand, Stekol (258) recently isolated N-acetyl-S-benzyl-cysteine from the urine of dogs to which he had fed benzyl chloride:



This is the only mercapturic acid so far obtained in which the sulphur of the acetylcysteine residue is not attached directly to a carbocyclic ring.

Phenols. When phenol, $C_6H_5 \cdot OH$, is administered to an animal, part of it is conjugated, part of it is oxidized, and part of it is excreted unchanged. Baumann (12) was the first to recognize that phenol is converted to a sulphuric acid derivative in the animal body. He isolated this compound in the form of its potassium salt, $C_6H_5 \cdot O \cdot SO_3K$, and confirmed its structure by synthesis (13). Later it was shown that part of the phenol is excreted in combination with glucuronic acid (144). This derivative, $C_6H_5 \cdot O \cdot C_6H_9O_6$, is a β-glycoside (223, 182). It is known that phenolic compounds are formed as a result of the action of intestinal bacteria on protein decomposition products (35, 277, 14), and it is generally accepted that these compounds give rise to part of the ethereal sulphates present in normal urine (148). It was long considered that the production of ethereal sulphate takes precedence over glucuronide formation in the detoxication of phenols. This is not supported by the observations of certain workers (157, 222) who have

reported the presence of glucuronide in urines containing considerable amounts of inorganic sulphate. It is unfortunate that few quantitative data are available concerning the extent to which glucuronide formation takes place in the detoxication of phenols, for there is evidence (268) that man excretes more phenol in conjugation with glucuronic acid than with sulphuric acid. In the dog, phenol is oxidized to some extent to quinol and catechol (18) and these derivatives are excreted as ethereal sulphates (18) and as glucuronides (144). The methyl substituted phenols tend to be oxidized to the corresponding carboxylic acids, e.g., p-cresol, $HO \cdot C_6H_4 \cdot CH_3$, is partly converted to p-hydroxybenzoic acid, $HO \cdot C_6H_4 \cdot COOH$ (196). The extent to which phenolic compounds undergo conjugation in the organism varies considerably with the nature and position of substituent groups in the carbocyclic nucleus. Williams (283) has studied the excretion of ethereal sulphates by the rabbit after the administration of phenol and a number of its mono-substituted derivatives. Compared with phenol itself, decreased conjugation is observed when an acidic group is present in the o-position, and increased when a basic group is present in this position. In the case of a neutral group in the o-position, the extent to which conjugation occurs is similar to that observed with phenol. The m-derivatives yield similar, though less marked, results to those obtained with the corresponding o- substituted derivatives of phenol. Although in most cases substitution in the p-position has little effect on the amount of ethereal sulphate formed, sulphate conjugation is markedly decreased by a p-COOH group and increased by a p-$CONH_2$ group. In these experiments no derivative tested was found to undergo sulphate conjugation to the extent of more than 40 per cent.

Following the development and application of their colorimetric method for determining urinary phenols, Folin and Denis (71) reported that a considerable proportion of the phenols present in urine is in the unconjugated form. In the urine of a patient undergoing benzene treatment for leukemia they found that the free phenols predominated. They stated that in normal urine 30–90 per cent. of the total phenols are in the free form, and Dubin (60), who used their method for determining phenols, reported similar results. In considering these findings it is necessary to recognize that the Folin-Denis "phenol" reagent gives a blue colour not only with phenols, but also with aromatic hydroxy-acids and other substances present in urine (267). By using a method for determining phenols which involved ether extraction of the

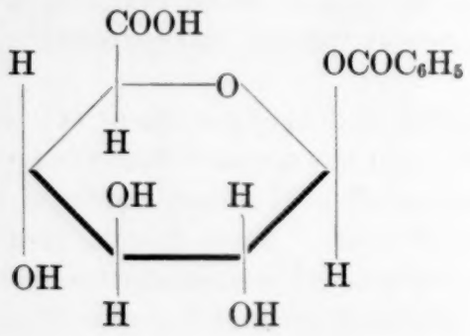
urine, Tisdall (267) obtained results for the excretion of total phenols in normal urine which are at least 50 per cent lower than those reported by Folin and Denis. He also observed that only a very small proportion of the volatile phenols (phenol and p-cresol) is present in the free state.

The work of Marrian and his co-workers (44, 42, 43, 188) and of Venning and Browne (273) has revealed that the process of excretion in conjugation with glucuronic acid is not restricted to the detoxication of foreign organic compounds. From human pregnancy urine Cohen and Marrian (43) have isolated oestriol glucuronide, and Venning and Browne (273) have obtained pregnandiol glucuronide. In the former compound the glucuronic acid is apparently not linked to the phenolic hydroxyl of the oestriol (43). There is present in mare's urine a combined oestrogen which appears to be a sulphuric acid derivative (227).

Carboxylic acids. Since considerations of space make it impossible to review here the vast amount of work which has been published on the detoxication of carboxylic acids, the present review has been based on the more recent investigations on this subject. For information on the earlier work in this field, the monograph by Dakin (56) and the reviews by Sherwin (234) and by Heffter (99) should be consulted.

Although benzoic acid has been found to undergo conjugation with glycine in every mammal so far tested, it is now known that in man (55, 183), the sheep (153), pig (51), dog (34, 200) and rabbit (84), it gives rise to a glucuronic acid derivative as well as hippuric acid. The structure of this derivative has been the subject of some controversy. Some workers (153, 197) were led to the conclusion that the compound is 1-benzoylglucuronic acid, whereas others (200, 213, 156) considered that the benzoic acid is attached to the glucuronic acid by an ester linkage to a carbon atom other than the aldehydic carbon atom. The question now appears to have been settled by the recent work of Goebel (82). As a result of the preparation of 1-bromo-2,3,4-triacetylglucuronic acid methyl ester (83), a pyranose derivative with the β-configuration (115), it is now possible to prepare derivatives of glucuronic acid substituted on the aldehydic carbon atom. By treating the above bromo-compound with silver benzoate, Goebel (82) prepared 1-benzoyl-2,3,4-triacetylglucuronic acid methyl ester and showed that this derivative is identical with the product obtained by acetylation of the methyl ester of the benzoic acid-glucuronic acid compound isolated from dog urine after feeding sodium benzoate. The benzoic acid

derivative which is excreted is therefore 1-benzoylglucuronic acid, and it has been assigned the structure:



The relative extent to which glycine and glucuronic acid are involved in the detoxication of benzoic acid varies considerably from species to species. In the dog, as much as 75 per cent of a dose of sodium benzoate may be excreted as benzoylglucuronic acid (203), whereas in man only 10–12 per cent of the dose is excreted in this form (205). The remainder of the dose in both cases is excreted almost entirely as hippuric acid. With reference to these species differences, Quick (206) has studied the formation of hippuric acid and benzoylglucuronic acid in the animal body from the standpoint of the law of mass action, and has concluded that the differences observed in the output of these substances depend on the factors which influence a reversible reaction. The reactions involved are:

Benzoic acid + glycine $\rightleftharpoons$ hippuric acid + water

Benzoic acid + glucuronic acid $\rightleftharpoons$ benzoylglucuronic acid + water.

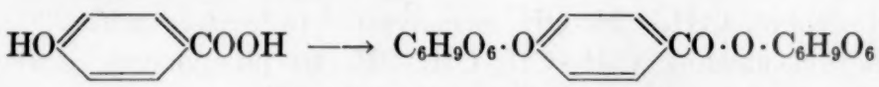
They are related in that benzoic acid is common to both of them, and Quick considers that the basic factors which determine the partition of benzoic acid between them are: (a) the rate of elimination of the end-products, hippuric acid and benzoylglucuronic acid; (b) the concentration and supply of glycine and of glucuronic acid; (c) the rates of the reactions between benzoic acid and glycine, and between benzoic acid and glucuronic acid.

It has frequently been observed that increases in the dose of a foreign compound fail to produce proportionate increases in the output of its detoxication products. For example, when increasing doses of sodium benzoate are administered to a dog on a diet containing little or no glycine, a point is reached beyond which further increases in the dose have little effect on the amount of hippuric acid excreted. On the other hand, the excretion of benzoylglucuronic acid continues to increase

with increasing doses of sodium benzoate far beyond the level at which the hippuric acid excretion becomes limited (199). It seems unlikely that conjugation with glucuronic acid is a secondary defence mechanism as was once believed, for in the dog, the formation of benzoylglucuronic acid appears to proceed at first more rapidly than the production of hippuric acid (203). Furthermore, it has been shown that in the rabbit benzoylglucuronic acid formation occurs with doses of sodium benzoate which are below those at which hippuric acid production is at a maximum (84).

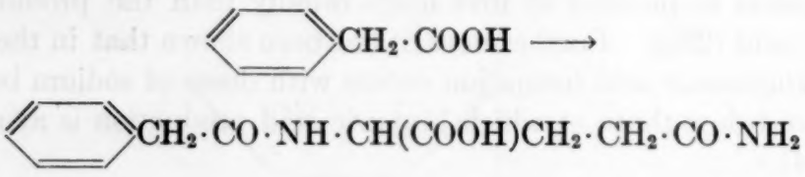
An example of the influence of the mode of administration of a foreign compound on the course of its detoxication is provided by the finding (87, 84, 271) that when sodium benzoate is injected intravenously into a rabbit, there is a greater excretion of unconjugated benzoic acid than is the case when the same dose of sodium benzoate is given orally.

The substituted benzoic acids frequently differ widely from benzoic acid in their tendency to undergo conjugation in the animal body. Thus it has been found that with the possible exception of certain o-halogen substituted acids (*c.f.* 109, 187, 208) the o-substituted benzoic acids do not combine to any marked extent with glycine in the organism. Quick (208) has shown that whereas this inhibitory action of the o-group on the conjugation of substituted benzoic acids with glycine is independent of the nature of the group, the output of glucuronic acid derivatives is decreased if there is an acidic group in the o-position, and increased if the group is a basic one. A considerable amount of attention has been paid to the detoxication of o-hydroxybenzoic acid (salicylic acid) because of the pharmacological action of this compound. Contrary to the findings of Bertagnini (24) and later workers, Hanzlik (95) concluded that it is improbable that salicylic acid is conjugated with glycine in the human or animal organism. Holmes (112) disagreed with this conclusion. More recently Quick (212) has been able to isolate salicyluric acid, $HO \cdot C_6H_4 \cdot CO \cdot NH \cdot CH_2 \cdot COOH$, in small amounts, from the urine of men receiving salicylic acid. Quick (209) has shown that the feeding of p-hydroxybenzoic acid to the dog leads to the excretion of an interesting compound in which the phenolic group and the carboxylic group of p-hydroxybenzoic acid are each conjugated with a molecule of glucuronic acid:

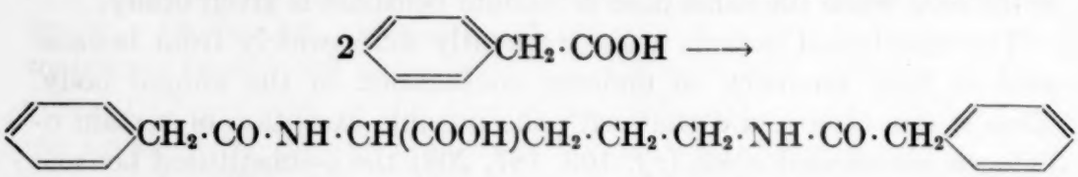


When p-hydroxybenzoic acid is ingested by man, it is excreted partly uncombined and partly conjugated with glycine (209).

In the human organism phenylacetic acid, unlike benzoic acid, does not undergo conjugation with glycine. As Thierfelder and Sherwin (264, 265) first showed, phenylacetic acid when ingested by man is largely excreted in combination with glutamine:



For a long time it seemed as though man alone is capable of bringing about this reaction. In the dog (224, 225), rabbit (225), cat (228), and monkey (233), phenylacetic acid is combined with glycine, and in the fowl (269) it is conjugated, as is benzoic acid (123), with ornithine:



Recently it has been found, however, that the chimpanzee can combine phenylacetic acid with glutamine (194). In view of these remarkable species differences, it is of interest to note that a phenylacetyl derivative of glycine, ornithine, or glutamine is largely excreted unaltered when administered to man, the dog, rabbit and fowl, irrespective of the type of conjugation which phenylacetic acid undergoes in the animal to which the derivative is given (241). Glucuronic acid is also involved in the detoxication of phenylacetic acid. In man, about 5 per cent of a moderate dose of this compound is excreted combined with glucuronic acid (7), and in the dog about one-third of the dose is eliminated in this form (203). Sherwin and his co-workers (39, 40, 169, 162, 163) have studied the fate of a wide variety of phenylacetic acid derivatives in man, the dog and the rabbit. Some of them are combined with glycine or glucuronic acid, many of them are largely excreted unchanged, but so far no compound other than phenylacetic acid has been found to undergo detoxication by union with glutamine.

Alcohols. The oxidation of primary aromatic alcohols to the corresponding carboxylic acids has been shown to occur in the body. Thus benzyl alcohol, $C_6H_5 \cdot CH_2 \cdot OH$, is converted to benzoic acid (230, 152), phenylethyl alcohol, $C_6H_5 \cdot CH_2 \cdot CH_2 \cdot OH$, to phenylacetic acid (56), and o-hydroxybenzyl alcohol, $HO \cdot C_6H_4 \cdot CH_2 \cdot OH$, to salicylic acid (175). In man and the rabbit, the oxidation of benzyl alcohol is rapid, and the

velocity of the oxidative reaction is at least as great as the rate of hippuric acid synthesis and excretion (245, 57). Some aromatic alcohols are converted to glucuronides in the body, as also are a number of aliphatic alcohols (180). This has been observed in the case of a primary alcohol, o-nitrobenzyl alcohol, formed in the body from o-nitrotoluene (124), and a secondary alcohol, phenylmethylcarbinol, formed after the administration of acetophenone (180, 262, 204). When triphenylcarbinol, $(C_6H_5)_3C \cdot OH$, is fed to rabbits, part of it can be recovered unchanged from the urine and feces (163).

Aldehydes. Most aromatic aldehydes are readily oxidized to carboxylic acids in the organism. Benzaldehyde, $C_6H_5 \cdot CHO$, given orally or by injection, is converted to benzoic acid (284, 46, 77), and in the dog may also yield small amounts of benzamide (45). The aldehydic group in other aromatic compounds such as vanillin (195) and o-, m-, and p-nitrobenzaldehydes (259), is also known to undergo oxidation in the body, and p-nitrophenylacetaldehyde, $NO_2 \cdot C_6H_4 \cdot CH_2 \cdot CHO$, in which the aldehydic group is in the side chain, is oxidized to p-nitrophenylacetic acid (237). From experiments with the aldehyde mutase of liver, it would appear that this enzyme does not act on aromatic aldehydes (189, 58).

In the presence of a suitable condensing agent, benzaldehyde and sodium acetate react *in vitro* to yield sodium cinnamate (Perkin's reaction) (192):

$$C_6H_5 \cdot CHO + CH_3 \cdot COONa = C_6H_5 \cdot CH{:}CH \cdot COONa + H_2O.$$

The fact that the heterocyclic aldehyde, furfuraldehyde, $C_4H_3O \cdot CHO$, undergoes an analogous change in the body to yield furfuracrylic acid, $C_4H_3O \cdot CH{:}CH \cdot COOH$ (127, 128), has naturally led to some speculation as to whether or not benzaldehyde is converted to cinnamic acid in the organism. No formation of cinnamic acid has been demonstrated, however, in experiments in which benzaldehyde has been given to animals by mouth or by injection (46, 77). Furfuracrylic acid can be oxidized by animals to pyromucic acid, $C_4H_3O \cdot COOH$ (72, 74), and it may be that if any cinnamic acid is formed from benzaldehyde, it is oxidized to benzoic acid. On the other hand, cinnamic acid formed from cinnamaldehyde, $C_6H_5 \cdot CH{:}CH \cdot CHO$, in the organism escapes oxidation to some extent and is excreted in combination with glycine (75).

Ketones. The mixed aliphatic-aromatic ketones tend to give rise to both secondary alcohols and carboxylic acids in the body. Acetophenone, $C_6H_5 \cdot CO \cdot CH_3$, is converted to phenylmethylcarbinol, $C_6H_5 \cdot$

$CH(OH)\cdot CH_3$, (excreted in conjugation with glucuronic acid), mandelic acid, $C_6H_5\cdot CH(OH)\cdot COOH$, and benzoic acid (177, 180, 262, 204). Benzylmethylketone, $C_6H_5\cdot CH_2\cdot CO\cdot CH_3$, is also oxidized to benzoic acid (53), and Dakin (53) has suggested that "It will probably be found that most aromatic methyl ketones primarily undergo oxidation in the body, so as to yield acids with two less carbon atoms, except in the case of acetophenone, in which the carbonyl group is directly attached to the nucleus." This is supported by the finding that phenylethylmethylketone, $C_6H_5\cdot CH_2\cdot CH_2\cdot CO\cdot CH_3$, (54, 108) gives rise to phenylacetic acid, $C_6H_5\cdot CH_2\cdot COOH$, as also does phenylbutylmethylketone, $C_6H_5\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot CO\cdot CH_3$, (108). This last compound would be expected first to yield phenylbutyric acid, $C_6H_5\cdot CH_2\cdot CH_2\cdot CH_2\cdot COOH$, which would then undergo β-oxidation to phenylacetic acid.

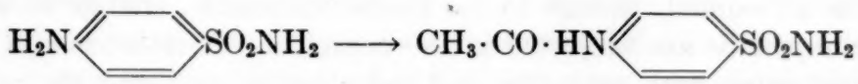
It has been observed (262) that a number of the aliphatic-aromatic ketones of the phenyl and benzyl series behave similarly to the methylketones of these series in that they give rise to benzoic acid in the body together with their corresponding secondary alcohols.

Certain aromatic ketones which are also phenolic in character are excreted in conjugation with sulphuric acid and glucuronic acid (178). p-Hydroxybenzophenone, $C_6H_5\cdot CO\cdot C_6H_4\cdot OH$, is said to be excreted unchanged (231). After the administration of o-aminoacetophenone, $NH_2\cdot C_6H_4\cdot CO\cdot CH_3$, to the rabbit, anthranilic acid, $NH_2\cdot C_6H_4\cdot COOH$, and the glucuronic acid derivative of o-aminophenylmethylcarbinol, $NH_2\cdot C_6H_4\cdot CH(O\cdot C_6H_9O_6)\cdot CH_3$, are excreted (119). These products are analogous to those obtained from acetophenone.

Amino compounds. Whereas simple aliphatic amines such as methylamine are largely destroyed in the body (132), the carbocyclic derivatives of the $R\cdot CH_2\cdot NH_2$ series are converted to carboxylic acids. Thus benzylamine, $C_6H_5\cdot CH_2\cdot NH_2$ (166, 118), and β-phenylethylamine, $C_6H_5\cdot CH_2\cdot CH_2\cdot NH_2$ (90), are oxidized to benzoic acid and phenylacetic acid respectively. Similarly, p-hydroxyphenylethylamine (66) and β-indolethylamine (67, 81) are converted to the corresponding carboxylic acids. On the other hand, Richter (217) has shown that amines of the $RR'CH\cdot NH_2$ series such as ephedrine, benzedrine and methylisomyn, are excreted unchanged by man.

A number of aromatic amino compounds have been shown to undergo acetylation in the body. When sulphanilamide (p-aminobenzenesulphonamide) is administered to man and certain animals it is excreted

partly free and partly in a conjugated form (79, 155), and Marshall *et alia* (155) have shown that in the case of man and the rabbit, the conjugated form is mainly, if not entirely, p-acetylaminobenzenesulphonamide:



This reaction does not occur in the dog (155), however, and it is therefore interesting to note that o-, m-, and p-aminobenzoic acids are excreted unchanged by the dog, whereas the m- and p-isomers are converted to the acetylamino derivatives by man and the rabbit, and only the o-form is excreted unchanged (168). Furthermore, the dog does not acetylate any of the monoaminophenylacetic acids, but excretes the o- and m-isomers unchanged and conjugates the p-form with glycine (39, 40, 169). In the rabbit all three acids are acetylated (39, 40, 169). On the other hand, acetylation of side chain amino groups has been shown to occur in the dog in the case of γ-phenyl-α-aminobutyric acid, $C_6H_5 \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ (139), and S-benzyl-cysteine, $C_6H_5 \cdot CH_2 \cdot S \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ (258).

Certain aromatic amino compounds when administered to animals are oxidized to phenolic derivatives. Thus aniline, for example, does not appear to be acetylated, but is oxidized to p-aminophenol, $HO \cdot C_6H_4 \cdot NH_2$, and excreted in conjugation with sulphuric acid (229) and possibly with glucuronic acid (143). This formation of a phenolic group also takes place in the case of β-naphthylamine, $C_{10}H_7 \cdot NH_2$, a compound which has been shown to produce bladder tumours in rabbits and dogs (106, 116). From the urine of dogs receiving β-naphthylamine, Wiley (281, 282) has isolated the sulphuric acid derivative of 2-amino-1-naphthol, the identity of which he has established by synthesis. In man acetanilide, $C_6H_5 \cdot NH \cdot CO \cdot CH_3$, is oxidized to p-acetylaminophenol, $HO \cdot C_6H_4 \cdot NH \cdot CO \cdot CH_3$ (165). In the rabbit acetanilide gives rise to p-aminophenol, which is excreted in conjugation with sulphuric acid and with glucuronic acid (129). In connection with these reactions, it is of interest to note that the liver and certain other organs of a number of animals, including the rabbit, contain an acylase which hydrolyses acetanilide *in vitro*, and also that a liver suspension can bring about the oxidation of aniline to p-aminophenol (159).

Dimethylaniline, $C_6H_5 \cdot N(CH_3)_2$, has been found to undergo demethylation in the dog and the rabbit. In the dog it is completely demeth-

ylated and gives rise to o-aminophenol (113). After injection into the rabbit it is excreted as the glucuronide of N-methyl-p-aminophenol, $HO \cdot C_6H_4 \cdot NH \cdot CH_3$ (113a).

Nitro-compounds. The reduction of the nitro-group to the amino-group is a frequent, though by no means invariable, change to which nitro-compounds are subjected in the organism. Nitrobenzene, $C_6H_5 \cdot NO_2$, undergoes both oxidation and reduction in man and the rabbit, giving rise to p-aminophenol, $HO \cdot C_6H_4 \cdot NH_2$, (158). This compound is also formed in the body from p-nitrophenol, $HO \cdot C_6H_4 \cdot NO_2$, and aniline (158). The mono-nitrotoluenes are oxidized to the corresponding nitrobenzoic acids (122, 124). It was Jaffé (124) who first drew attention to the fact that o-nitrobenzoic acid, in contrast to the m- and p-isomers, is not excreted in conjugation with glycine. He also observed that o-nitrotoluene gives rise to a derivative of o-nitrobenzyl alcohol. On hydrolysis, this derivative yields an acid which, from Jaffé's account of its properties, is almost certainly glucuronic acid.

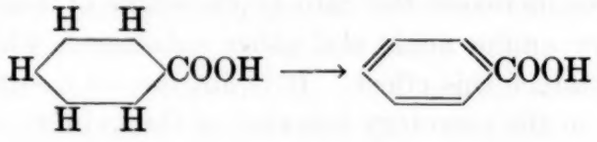
The nitrophenols tend to undergo reduction in the organism to aminophenols, and it has been suggested that their stimulatory effect on metabolism is related to this fact (94). Meyer (158) found that m- and p-nitrophenols are reduced in the rabbit to the corresponding aminophenols, but he was unable to find evidence of the occurrence of such a reduction in the case of o-nitrophenol. In the animal body, 2, 4-dinitrophenol is converted to 2-amino-4-nitrophenol and 2-nitro-4-aminophenol (89), and the nitro-group in position 2 of 2,4,6-trinitrophenol (picric acid) is said to undergo reduction, thereby forming picramic acid (276). Following the administration of the mono-nitrophenols, there is an increased ethereal sulphate excretion (16, 93, 283).

In man and the dog, the mono-nitrobenzaldehydes, $NO_2 \cdot C_6H_4 \cdot CHO$, are oxidized to the corresponding nitrobenzoic acids (259, 46, 237, 114) and here again it has been noted that whereas the m- and p-acids are excreted in conjugation with glycine, this does not occur in the case of o-nitrobenzoic acid. In the rabbit m-nitrobenzaldehyde undergoes oxidation, reduction and acetylation, and is excreted as m-acetylaminobenzoic acid, $CH_3 \cdot CO \cdot NH \cdot C_6H_4 \cdot COOH$ (46). The mono-nitrophenylacetic acids, $NO_2 \cdot C_6H_4 \cdot CH_2 \cdot COOH$, tend to be excreted unchanged by man and the rabbit; some conjugation occurs in the case of the m- and p-isomers in the dog (236, 39, 169).

Sulphonic acids. Such information as is available on the subject indicates that the sulphonic acid group, when attached to the aromatic nucleus, does not undergo modification in the body (221, 16, 136).

The increased excretion of ethereal sulphates which follows the administration of phenol and guaiacol does not take place when the sulphonic acid derivatives of these compounds are fed (16, 136).

Cyclohexane derivatives. The changes which foreign compounds of the hydroaromatic series undergo in the organism are of particular interest, in view of the physiological importance of a number of compounds which possess condensed carbocyclic structures of the reduced type. The fate of a number of cyclohexane derivatives in the body has recently been studied by Bernhard (23). He has shown, as also has Friedmann (73), that when hexahydrobenzoic acid, $C_6H_{11}\cdot COOH$, is administered to the dog, it is converted to benzoic acid:



He has also found that hexahydrohippuric acid, $C_6H_{11}\cdot CO\cdot NH\cdot CH_2\cdot COOH$, hexahydrophenylpropionic acid, $C_6H_{11}\cdot CH_2\cdot CH_2\cdot COOH$, and the N-methyl and N-dimethyl derivatives of the amide of hexahydrobenzoic acid, $C_6H_{11}\cdot CO\cdot NH_2$, undergo dehydrogenation and conversion to benzoic acid in the dog. When hexahydrophenylacetic acid, $C_6H_{11}\cdot CH_2\cdot COOH$, is administered, however, no formation of phenylacetic acid can be detected. When the hydroxy, methyl, o- and p-dimethyl, and amino derivatives of cyclohexane, C_6H_{12}, are administered, they are apparently destroyed or stored. Bernhard has also investigated the fate of the hexahydrotoluic acids, $CH_3\cdot C_6H_{10}\cdot COOH$, and has shown that although hexahydro-m-toluic acid is dehydrogenated to yield m-toluic acid, $CH_3\cdot C_6H_4\cdot COOH$, dehydrogenation is only partial in the case of the p-derivative, and apparently does not occur in the case of either the cis- or trans- form of hexahydro-o-toluic acid. From these and other findings, it appears that in the organism of the dog marked dehydrogenation of cyclohexane derivatives occurs when there is a free carboxyl group substituted directly in the ring, or when such a group can be formed. Other substituent groups can also influence dehydrogenation, for example, the o-substituted methyl group exerts an inhibitory influence.

SOURCE OF THE SUBSTANCES EXCRETED IN CONJUGATION WITH CARBOCYCLIC COMPOUNDS. *Glycine.* It is known that the amount of glycine available in the body for the detoxication of benzoic acid is in excess of that present in the diet. The origin of this additional glycine is uncertain. Some workers (154, 218) have concluded that it is derived from "extra

destroyed" protein, and not from protein which would be metabolised were no benzoic acid given. It is now generally accepted, however, that the formation of hippuric acid in man and animals can involve a synthesis of glycine from nitrogen which would normally be excreted as urea (280, 150, 147, 242, 260). Experiments with both growing (51, 85, 86) and adult (87) animals have revealed that the rate at which the organism can produce glycine for detoxicating purposes is distinctly limited, and in a fasting man it is this factor which eventually limits the output of hippuric acid when increasing doses of sodium benzoate are ingested (205). Griffith and Lewis found that the administration of glycine (87), or protein digests rich in glycine (88), to rabbits receiving sodium benzoate increases the rate of excretion of hippuric acid. A number of other amino acids and other substances which have been tested fail to produce this effect. It is not due to a stimulatory influence of glycine on the excretory function of the kidneys, on the absorption of benzoate from the intestine, or on general metabolism. Griffith and Lewis (87) have suggested that this increased rate of excretion of hippuric acid is due to a greater rate of synthesis of this compound resulting from the presence of increased amounts of preformed glycine in the organism. It has also been observed that when glycine is fed to a dog (206) or a rabbit (271) receiving phenylacetic acid, the rate of excretion of phenaceturic acid is increased.

Glutamine. Under normal conditions a man can convert considerable amounts of phenylacetic acid to phenylacetylglutamine (239, 7), e.g., about 95 per cent of a dose of 10 grams of phenylacetic acid is excreted in this conjugated form (7). Shiple and Sherwin (242) have shown that glutamine can be synthesized by man for detoxicating purposes at the expense of urea nitrogen.

Ornithine. A fowl on a non-nitrogenous diet can apparently synthesize ornithine for the detoxication of benzoic acid, and this results in a decreased excretion of uric acid. The output of ornithuric acid by the fowl is increased by the administration of arginine (50).

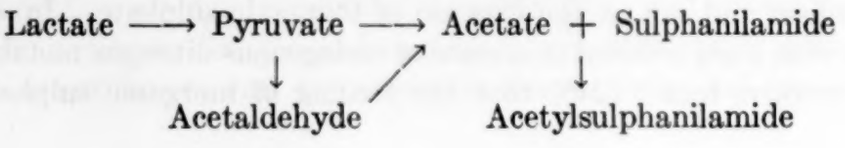
Cysteine. Before the recent work of Stekol (250) on the determination of mercapturic acids in urine, anyone wishing to make a quantitative study of the excretion of p-bromophenylmercapturic acid, for example, was obliged either to isolate and weigh the acid or to assume that increases in the neutral sulphur of the urine after bromobenzene administration are due to mercapturic acid. It is well known that the methods available for the isolation of mercapturic acids are by no means quantitative, and in certain instances their use has yielded

misleading results (*c.f.* 1, 2, 151). On the other hand, certain workers (8, 279, 250, 252) have questioned the validity of the assumption that mercapturic acid formation is alone responsible for the increases in the neutral sulphur of the urine after giving compounds of the type of bromobenzene to animals. This assumption has been criticised (252) on the ground that certain sulphur compounds on ingestion may cause increases in the neutral sulphur of the urine which are independent of their effect on mercapturic acid formation. In the light of these considerations it is not surprising that the literature reveals some differences of opinion on the subject of the formation of mercapturic acids *in vivo*. Although early investigators (266, 133, 170) emphasized the importance of exogenous cystine in the synthesis of mercapturic acids, it now appears that the animal can use tissue sulphur for this purpose, for mercapturic acid formation has been shown to occur in various fasting animals, including the rabbit (185), cat (275), and the growing (248) and adult dog (3). The work of White and Lewis (279) on the influence of l-cystine and dl-methionine on the sulphur excretion of dogs receiving bromobenzene on a low sulphur diet, first suggested that these two amino acids behave similarly in the detoxication of bromobenzene, and it is now known that under the above conditions they both bring about an increased mercapturic acid formation (252). The growth of young rats on a basal diet in which the level of cystine and methionine is just sufficient for good growth can be inhibited by giving bromobenzene (278). The most probable explanation of this effect is that the detoxication of bromobenzene takes precedence over growth requirements under these conditions, for resumption of growth can be induced either by stopping the administration of bromobenzene (278, 91) or by the addition of cystine or methionine to the basal diet (278). Similar results have been obtained by Stekol (256). Stekol, who has made a detailed study of mercapturic acid synthesis in animals (250–258), has been led by his findings to suggest (255, 257) that the extent of mercapturic acid formation is determined by the sulphur of the diet only in so far as this influences the nutritional state of the animal.

Sulphuric acid. There has been some disagreement among various workers concerning the source of the sulphur involved in the formation of ethereal sulphates in the organism. Muldoon *et alia* (170) considered that ethereal sulphates are formed as a result of endogenous metabolism and not at the expense of inorganic sulphate. In experiments with a pig reduced to a state of endogenous nitrogen metabolism, these workers found (240) that the feeding of inorganic sulphate to-

gether with bromobenzene, phenol, or p-chlorophenol resulted in no increase in the excretion of ethereal sulphates. It has since been found that the pig does not readily form ethereal sulphates (49, 251) and it seems probable that it would be difficult to demonstrate a combination of phenolic compounds with inorganic sulphate in this animal. Sherwin and his co-workers (219, 220) obtained evidence that mercapturic acids can be oxidized in the organism, and suggested (240) that ethereal sulphates arise partly as a result of the oxidation of mercapturic acids formed from phenols. Other workers (38, 145) have failed to find evidence of the conversion of mercapturic acids to ethereal sulphates in the organism. Because of this, and from structural considerations, Hele (101, 49) has criticised the view that mercapturic acid formation is a stage in ethereal sulphate production and has suggested that König's (142) work would make it seem more probable that oxidation of mercapturic acids in the body would lead to sulphonic acid formation. Hele found that administered sodium sulphate is utilized by the dog for the formation of ethereal sulphates when guaiacol carbonate (100), phenol or indole (102) are given, and suggested (102) that "in every case ethereal sulphate, whether arising endogenously or exogenously, is formed by the union of the sulphate ion with phenolic compounds."

Acetic acid. Quantitative studies (107, 97) on the acetylation of p-aminobenzoic acid in the rabbit have shown that the excretion of p-acetylaminobenzoic acid is markedly increased by the administration of a variety of substances, including sodium acetate, pyruvic acid, and ethyl acetoacetate. Furthermore, the injection of insulin also has this effect (96). The acetylation of sulphanilamide by tissues *in vitro* has been studied by Klein and Harris (135). They showed that the acetylation of this compound can occur in slices of rabbit and human liver, and that, normally, the factor limiting the reaction under these conditions is the rate of acetate production by the tissue. Addition of acetate increases the amount of conjugation as also does the addition of substances which give rise to acetate in the tissue. The decreased acetylation which is observed, in the absence of added acetate, under anaerobic conditions or in the presence of low concentrations of arsenious oxide or iodoacetamide, is probably mainly due to an inhibition of acetate formation. Klein and Harris (135) suggest the following scheme for the acetylation of sulphanilamide by rabbit liver:



In the light of theories (274, 139, 137) concerning the rôle of acetylation in the synthesis of amino acids in the animal body, Stekol (258) has suggested that mercapturic acid formation may be the result of an interaction of ammonia, pyruvic acid, and an α-keto-β-thiopropionic acid derivative, rather than the result of direct acetylation of a cysteine derivative.

Glucuronic acid. Fischer and Piloty (68) advanced the theory that conjugated glucuronic acids arise in the organism as a result of a combination of a hydroxy-compound with glucose to yield a glucoside which undergoes oxidation to the corresponding glucuronic acid derivative. Although the reported conversion in the body of certain glucosides to their corresponding glucuronides (110, 111, 92) may be interpreted in favour of this theory, the possible hydrolysis of the glucoside followed by the conjugation of the hydroxy-compound with glucuronic acid has to be taken into consideration. Support for this possibility has come from the finding (198) that phenyl-α- and β-glucosides are hydrolysed when administered orally or subcutaneously to the rabbit, and behave like the equivalent amount of phenol with respect to the amount of ethereal sulphate to which they give rise. The production of glucuronic acid derivatives of borneol or benzoic acid by the fasting depancreatised dog occurs as readily as in the normal dog and results in a decrease in the output of urinary sugar which corresponds to the amount of glucuronic acid excreted (201). When insulin is injected into normal dogs receiving sodium benzoate, the production of glucuronic acid is increased (210). Quick's observations have led him to suggest (202) that glucuronic acid can be produced by the animal both from carbohydrate and from protein. Miller *et alia* (160, 161) prefer to take the view that glucuronic acid liberated by the digestion of mucin is used for detoxication purposes. The findings of Ambrose and Sherwin (9) do not support this view, and they believe that the glucuronic acid used in detoxication is mainly synthesised from glycogenic substances. It has been noted that the administration of certain drugs to pentosuric subjects leads to an increased excretion of l-xyloketose, and the suggestion (64) that this is related to the formation of glucuronic acid in response to these drugs has received support from the finding that the ingestion of d-glucuronic acid by these individuals also increases the excretion of pentose (65). The feeding of mucin has been shown to have no significant effect on the excretion of l-xyloketose (65).

SITE OF DETOXICATION. Information concerning the site of detoxication has been mainly derived from observations on the extent to

which the detoxication of a given compound occurs in: *a*, animals suffering from injury, exclusion or extirpation of an organ under investigation; *b*, perfusion of isolated organs; *c*, experiments with tissue slices or ground tissues. While there is still much uncertainty concerning the extent to which any one tissue is responsible for the detoxication of a given compound, it has become generally accepted that the rôle of the liver in detoxication is a major one. The kidney may also play an important part in certain detoxication reactions. Thus it appears, for example, that in the dog the kidney is the sole site of synthesis of hippuric acid. This follows from the work of Bunge and Schmiedeberg (36). Although this work has been criticized (134), it has been supported by the findings of other investigators (140, 10, 244, 207). It is interesting to note that in the dog the synthesis of phenaceturic acid can occur elsewhere than in the kidney, for phenaceturic acid has been isolated from the blood of a nephrectomized dog following the injection of phenylacetic acid (207). The oxidation of phenyl derivatives of fatty acids has been found to take place in the kidney of the dog. Snapper and Grünbaum (243) showed that the perfusion of dog kidney with blood containing glycine and phenylpropionic acid or phenylvaleric acid results in the formation of hippuric acid, whereas phenylbutyric acid gives rise to phenylaceturic acid. Although there is evidence that the kidneys of certain animals, other than the dog, are capable of bringing about the synthesis of hippuric acid (244), the kidney is not invariably the site of formation of this compound. Hippuric acid synthesis has been observed in nephrectomized rabbits (226, 207) and also as a result of the perfusion of rabbit liver with blood containing sodium benzoate (76). Quick (207) attributes this synthesis to an enzyme, hippuricese, and has concluded that whereas this enzyme is confined to the kidney in the dog, this is not so in the rabbit and in man. Quick (205, 211, 214) has shown that the rate of excretion of hippuric acid by normal persons after the ingestion of sodium benzoate is approximately constant, and that the rate is lowered in the case of persons suffering from certain types of hepatic disease. He regards this reduced rate of excretion of hippuric acid as being due primarily to a decrease in the ability of the liver to synthesise glycine and partly to injury to the enzyme system which brings about the conjugation of benzoic acid with glycine. On the basis of these considerations Quick has developed a liver function test, the clinical value of which he has studied in detail (211, 214). Other workers have confirmed the finding that there is a decreased output of hippuric acid in certain pathological conditions of the liver

produced by disease (272, 246, 141) or by poisoning with chemical agents (131, 6, 270).

There is a considerable amount of evidence which indicates that the formation of ethereal sulphates and glucuronides takes place in the liver (12, 41, 62, 63, 190, 186). Hemingway, Pryde and Williams (105) have recently studied the question of the site of glucuronic acid conjugation by applying a perfusion technique to various combinations of dog tissues. In experiments with a pump-lung-kidney preparation of the type described by Hemingway (103, 104) they showed that the isolated kidney could excrete glucuronides but could not form them. When this preparation was used in conjunction with a liver perfused in a manner similar to that described by Bauer *et alia* (11), it was found that the addition of phenol, chloral hydrate, camphor or borneol to the perfusing blood resulted in the presence of increased amounts of glucuronide in the urine. Other organs (spleen, hind-limb) were perfused with blood containing phenol, but only in those preparations in which the liver was perfused was evidence of glucuronide synthesis obtained. The importance of the liver in the formation of glucuronides is further emphasized by the decreased excretion of these compounds which has been observed in cases of hepatic disease (26, 173, 174) and poisoning of the liver by chemical agents (26, 193, 172). Recent studies by Fishman (69, 70) on β-glucuronidase have revealed that this enzyme is present in various organs of the dog, and that the amounts present increase after the feeding of large amounts of borneol. If, as seems possible, β-glucuronidase possesses synthetic activity, such a finding would suggest that the liver is not the only tissue involved in glucuronide formation. Lipschitz and Büding (149) have observed the formation of glucuronide in the presence of borneol in liver slices, and to a lesser extent in kidney slices.

The perfusion of dog liver with blood containing α-aminophenylacetic acid has been shown (181) to result in the conversion of this compound to its acetylamino derivative. Muenzen *et alia* (167) have concluded from their investigations that the acetylation of foreign compounds is probably confined to the liver. In their studies on the acetylation of sulphanilamide by tissues *in vitro*, Klein and Harris (135) were able to demonstrate the occurrence of this reaction in experiments in which they employed intact liver cells.

As the foregoing examples show, much evidence has accumulated which indicates that the liver is able to bring about the synthesis of conjugated derivatives of a variety of foreign compounds. When

these compounds are absorbed from the gastro-intestinal tract, it seems probable that the liver, by virtue of its position, plays a major part in their detoxication. With the exception of the kidney, little is known at present concerning whether or not tissues other than the liver are capable of participating in the detoxication of carbocyclic compounds.

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COENZYMES

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Most enzymes are heat-labile, non-dialyzable, proteinaceous compounds of high molecular weight, which have been produced by living cells, and which function as catalysts in specific chemical reactions. Coenzymes, like enzymes, are catalysts produced by the living cell, and are necessary in enzymic reactions, but they are compounds of lower molecular weight, heat stable, and dialyzable. Though both enzymes and coenzymes are elaborated by the living cell, they may be separated from it and studied in vitro.

The term coenzyme was introduced in 1897 by Bertrand, who applied it to calcium and to manganese salts necessary for the action of certain plant enzymes such as pectase and laccase (1). In 1904 Harden and Young found that the thermostable, dialysable fraction of boiled yeast juice greatly increased the fermentation of cell-free extracts (2, 3). They termed their active substance the coenzyme of yeast juice. Since the proteinaceous enzyme-complex of yeast was called "zymase," the coenzyme of yeast juice came to be called cozymase (4).

The earlier workers believed that coenzymes "activated" enzymes, and many other examples of coenzyme activity were reported, such as the activation of plant diastase by asparagin (5), the action of inorganic chlorides on pancreatic diastase (5), the action of calcium on the coagulation of casein by rennin (5). Sodium cholate and glycocholate also showed "coenzyme activity" (6). A coenzyme for the urease of the soy bean was reported (7), though pure crystalline urease does not require a coenzyme (8).

In 1918 Meyerhof made the important observation that the coenzyme of alcoholic fermentation (Harden and Young's coenzyme) occurs in muscle, animal organs, and milk, as well as in yeast (9). The most active preparations were made by extracting frog muscle with boiling water. This was the first preparation of muscle "Kochsaft," which has been used so extensively in coenzyme research. Meyerhof further observed that this "boiled juice" greatly increased the respiration of

yeast and of minced muscle. Thus, the coenzyme of yeast fermentation became linked with muscle respiration.

For several years Szent-Gyorgyi and co-workers studied a coenzyme connected with the oxidation of lactic acid by lactic dehydrogenase (10–13). This coenzyme was usually obtained from muscle "Kochsaft" and was also a thermostable, dialysable substance. Later work has shown that the lactic coenzyme is identical with cozymase, and that it functions not only in the oxidation of lactic acid but also in the oxidation of several other naturally occurring compounds.

In 1932 Warburg constructed an enzyme system in which hexose monophosphate (Robison ester) could be oxidized in vitro by molecular oxygen (14–16). The system consisted of a dehydrogenase (Zwischensubstanz) from yeast, a coenzyme from red blood cells, and the yellow enzyme (flavo-protein), in addition to oxygen and the substrate. Thus Warburg explained the earlier observation, that methemoglobin present in red blood cells oxidized carbohydrate whereas methemoglobin in solution failed to do so (17, 18). For oxidation both the phosphorylating mechanism and the coenzyme present in the red blood cells were essential. Warburg later succeeded in isolating the coenzyme and characterizing it as a dinucleotide (16). It consists of one molecule of adenine, one of β-nicotinic acid amide, three of phosphoric acid, and two of pentose. Warburg further demonstrated that his coenzyme could be reversibly oxidized and reduced, thus indicating the nature of its catalytic action. The coenzyme was reduced by hexose monophosphate in the presence of the appropriate dehydrogenase by "accepting" two atoms of hydrogen. This reduced coenzyme was reoxidized by the transfer of two atoms of hydrogen to the yellow enzyme. The yellow enzyme, thus reduced, was reoxidized by molecular oxygen with the formation of hydrogen peroxide. It was shown that the pyridine ring of the coenzyme actually accepted and gave up the hydrogen.

The accurate characterization of cozymase quickly followed the characterization of Warburg's co-ferment, and it, too, was found to contain nicotinic acid amide, the pyridine ring of which could be reversibly reduced and oxidized (19–21). Thus, both the composition and the mode of action of two coenzymes were established.

Auhagen, in 1932, reported that a coenzyme was necessary for the action of carboxylase (22), an enzyme which converts pyruvic acid into acetaldehyde, and in general converts α-keto carboxylic acids into the corresponding lower aldehydes by decarboxylation. Remarkable progress has been made in the study of this coenzyme, cocarboxylase. It

has been isolated and synthetized and found to consist of thiamin (vitamin B_1) and two phosphoric acid groups.

In 1936 Das (23, 24), working in Euler's laboratory, demonstrated that the soluble amino-acid-oxidase of Krebs (25) requires a coenzyme for its activity. Further work from Warburg's laboratory and by Straub, working in Keilin's laboratory, indicated that this coenzyme differs chemically from those previously discussed in that it contains flavin in addition to adenine, pentose, and phosphoric acid. This review will deal mainly with the above coenzymes, although certain other substances must be mentioned since they satisfy the basic criteria for coenzymes, being heat stable, dialyzable, and active in enzyme reactions; namely, adenosine triphosphate, glutathione, and coenzymes R and T. The current tendency is to reserve the term "coenzyme" for organic substances. Inorganic ions necessary in enzyme reactions are called "inorganic activators" or simply "necessary inorganic ions."

The Pyridine Nucleotides. Nomenclature. Each of these coenzymes has been referred to by many names (26, 27). Diphosphopyridine nucleotide, Harden and Young's coenzyme, was named cozymase (4) and later codehydrase 1 (28), by Euler and his associates. Other terms used are coenzyme 1, fermentation coenzyme, yeast coenzyme, and lactic acid coenzyme. Probably the most commonly used terms are cozymase and coenzyme 1; however, since the chemical composition is known, it seems best to refer to it as the diphosphopyridine nucleotide, or the abbreviation DPN.

Triphosphopyridine nucleotide was originally called the "hydrogen carrying coenzyme of red blood cells" (16). Other names for this coenzyme have been Warburg's coferment, coferment, codehydrase 2, coenzyme 2, blood coenzyme and hexose monophosphate coenzyme (26, 29). Again, the chemical name or its abbreviation "TPN" is to be preferred.

Occurrence. Diphosphopyridine nucleotide was originally isolated from yeast, which still is the best source (3, 30). It is present in all varieties (31), but the content varies with species and with the condition of culture (27, 31–33). The concentration is fairly high in certain microörganisms, such as T. helviticum (34), and Strep. lactis (31). In others, such as Penicillium glaucum (31), or B. aerogenes (35), little if any is present. DPN is found in practically all animal tissues (27). Muscle, liver and kidney contain large amounts (15, 36–38), as do red blood cells (16, 32) and retina (36). Relatively little is found in blood serum, bone, bone marrow, lymph nodes, or connective tissue

(32, 36). The gray matter of the brain contains much more than the white matter (36). In the adrenals the concentration is highest in the cortex (36). There is considerable species variation, the blood of birds, for example, containing much more than the blood of mammals (29, 32). It has been found in invertebrate muscle (39) and in tumors (40). Small amounts of DPN are present in certain mushrooms (41), and in the seeds or root-tips of certain higher plants (27, 42, 43).

Triphosphopyridine nucleotide was discovered in red blood cells (18). It has also been isolated from them (16), from heart muscle and from yeast (44). Later work has indicated it to be very widely distributed in cells (29), and Warburg claims it to be a "ubiquitous cell constituent" (45).

Determination of diphosphopyridine nucleotide. 1. *Euler and Myrbäck* (30, 46–48). The most widely used method for the determination of diphosphopyridine nucleotide involves an artificial fermenting system in which all the necessary factors are present except the coenzyme. When DPN is added, the system ferments, and the CO_2 evolved is a measure of the amount of coenzyme present. The CO_2 is measured manometrically in a Warburg apparatus. A unit of cozymase is that amount which will liberate 1 cc. of CO_2 per hour under standard conditions. The purity of a preparation equals the units per gram of dry weight. A typical determination contains the following: 0.3 gram "apozymase,"[1] 0.1 gram glucose, 0.15 mol. phosphate, 5 mgm. hexose diphosphate, DPN solution and water to make 2 cc.

The apozymase is a washed yeast preparation containing a mixture of enzymes necessary in fermentation. It is prepared as follows: Pressed yeast is dried in air, then suspended in water and washed many times. The pasty mass is removed and dried on a glass plate. It has no action in the absence of DPN, and certain preparations are also deficient in Mg.

2. *Warburg's "Gär test"* (20). The chief criticism of the Euler-Myrbäck method is the uncertainty involved in the use of crude materials such as "apozymase." Warburg's method involves the use of purified materials. The reacting mixture contains (a) proteins A and B from yeast prepared according to Negelein (49); (b) inorganic salts Mn, Mg, NH_4; (c) substrates hexose monophosphate (Robison ester), H_3PO_4, and acetaldehyde; (d) the coenzymes diphosphopyridine nu-

[1] According to v. Euler's nomenclature the entire enzyme-complex needed for a reaction is called the "holoenzyme", i.e., holozymase. That which is left after removal of substrate and coenzyme is called the "apoenzyme", i.e., apozymase.

cleotide and adenosin triphosphate. Both are needed for a reaction. When either is present alone, the test can be used for the detection of the other.

The reaction is carried out in 0.3 per cent $NaHCO_3$ solution in a standard Warburg flask, and the gas space is filled with an inert gas containing 10 per cent CO_2. (Warburg uses argon.) The chief reaction involved is the reduction of acetaldehyde to ethyl alcohol, with a concurrent break-down of the hexose monophosphate. The over-all reaction appears to be:

$$\text{hexose monophosphate} + 2\ CH_3\ CHO + H_2O \rightarrow$$
$$CH_3\ CO\ COOH + CH_2OH_2PO_3\ CHOH\ COOH + 2\ CH_3CH_2OH$$

The production of pyruvic and phosphoglyceric acids results in an increase in the acidity of the solution causing the liberation of CO_2 from the bicarbonate, in amounts proportional to the activity of the coenzyme present. The gas is measured manometrically.

The Warburg test differs from the Euler-Myrbäck test in several particulars: the Euler-Myrbäck reaction attempts to duplicate the entire fermentation process; the Warburg reaction involves only a part of the process. In the Euler reaction the CO_2 evolved comes from the breakdown of the substrate; in the Warburg test, the CO_2 evolved is an index of the change in acidity of the mixture.

3. *Chemical determination of the pyridine nucleotides.* Both diphosphopyridine nucleotide and the triphosphopyridine nucleotide react similarly in this determination. The coenzyme as isolated (oxidized form) does not fluoresce in ultraviolet light, nor does it have an absorption band in the near ultraviolet. On the contrary, the reduced coenzyme exhibits a white fluorescence in ultraviolet light, and has a pronounced absorption band with a maximum at 340 mμ (16, 20). Thus it is possible to determine the amount of coenzyme present in a material by first reducing it, either enzymatically or by means of chemicals such as sodium hydrosulfite, and then quantitatively determining the amount of fluorescence, or the change in absorption at 340 mμ (16, 20, 50, 51).

These coenzymes can also be determined by completely reducing the pyridine ring with hydrogen in the presence of Pt. A borate buffer is used. In this reaction one molecule of coenzyme absorbs 6 atoms of hydrogen (16, 52).

Determination of triphosphopyridine nucleotide. TPN is determined by measuring the rate of oxygen absorption in a system in which it is

the limiting factor, the materials present being hexose monophosphate, dehydrogenase, yellow enzyme, and oxygen in addition to the coenzyme (16). If the other materials are present in excess, the rate of oxygen uptake is proportional to the amount of TPN present.

Preparation of diphosphopyridine nucleotide. (Method of Euler and Myrbäck (30).) Washed top or bottom yeast with a cozymase value (A Co) of 100 units is added to 4 volumes of water at 75 to 80°C. with stirring. The mass is filtered through linen, or centrifuged, and the filtrate concentrated in vacuo at 60°. The material is then dialyzed for 5 hours through parchment, 80 per cent of the coenzyme passing into the dialysate, which has a strong green fluorescence. The solution is treated with lead acetate, the precipitate filtered off, suspended in water, and decomposed with H_2S. Further purification is effected by absorbing on freshly precipitated $Al(OH)_3$ and diluting with H_3PO_4. The coenzyme can be precipitated with $HgNO_3$, picric acid, or phosphotungstic acid. The product obtained by the above procedure consists of diphosphopyridine nucleotide plus large amounts of adenine nucleotide.

For other methods of preparation see 21, 53–57 and especially 58.

Preparation of both pyridine nucleotides. (Method of Warburg (19, 20).) This method yields three coenzymes in pure form, diphosphopyridine nucleotide, triphosphopyridine nucleotide, and adenylic acid. Washed red blood cells are cytolyzed in distilled water, and the proteins precipitated with acetone. The solution is centrifuged, the liquid phase concentrated in vacuo, and treated with mercury acetate. The precipitate contains the three coenzymes. It is decomposed with H_2S, and the nucleotides precipitated with acetone. One thousand liters of red blood cells yielded 48 grams of raw nucleotides (20).

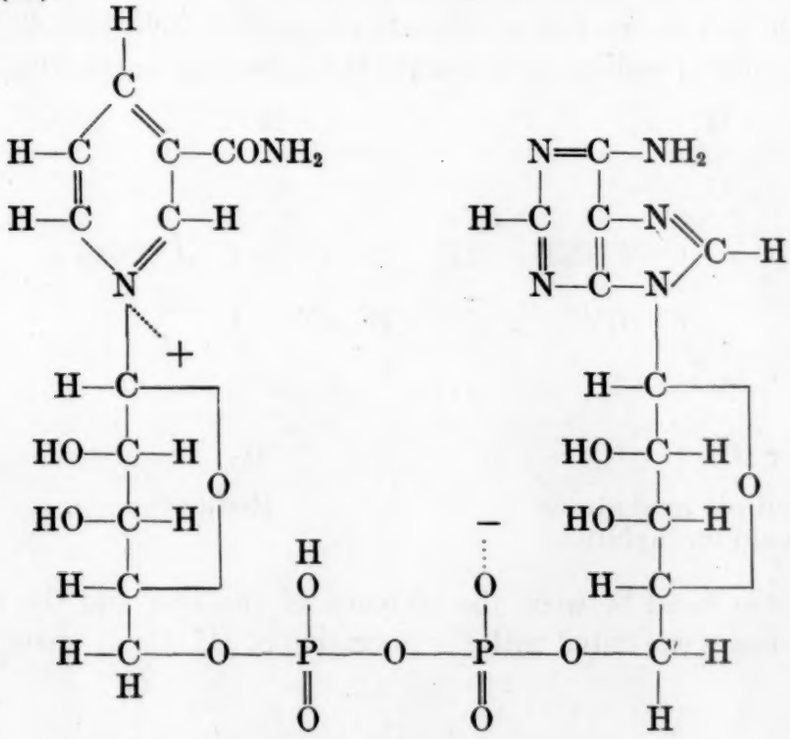
The separation of the nucleotides depends on the unequal solubilities of their barium salts. The barium salt of adenine nucleotide is insoluble in water, the barium salt of triphosphopyridine nucleotide is insoluble in dilute alcohol, whereas the barium salt of diphosphopyridine nucleotide is soluble in dilute alcohol. After separation, each fraction is then subjected to further purification. DPN is precipitated with mercury acetate, followed by precipitation with acetone, and finally by precipitation with ethyl acetate out of acid methanol solution.

Chemistry of the pyridine nucleotides. The pyridine nucleotides (DPN, TPN) are soluble in water, stable at 80°C., and readily diffuse through membranes (3, 59, 60). Their molecular weight is about 700. They are insoluble in the fat solvents, and can be precipitated by them, for example, by the addition of ethyl acetate to an aqueous methanol

solution (16). These coenzymes readily form salts with Ba, Pb, Hg, Ag, and Zn, (16, 21, 53–56, 59, 61–63) many of the salts being insoluble in water or in dilute alcohol. The coenzymes can also be precipitated with phosphotungstic acid (59) or tannic acid (59, 64). Aqueous solutions of the pyridine nucleotides are acid in reaction (16) and in general the oxidized forms are much more stable in acid than in alkali (16, 20, 65–68). The reverse is true of the reduced forms (16, 19, 50, 57, 65, 69, 70). The coenzymes are rapidly destroyed by ultraviolet light (71).

The coenzymes can be absorbed on Al_2O_3(61–63), triphosphopyridine nucleotide being held more firmly than diphosphopyridine nucleotide (58). This makes it possible to separate the 2 coenzymes by chromatographic means, since they can be eluted with H_3PO_4 (72). The 2 nucleotides can also be separated by virtue of the unequal solubility of their barium salts in dilute alcohol (16).

The structure of the pyridine nucleotides has been established by Warburg (16, 20) and Euler (72) and their co-workers. Diphosphopyridine nucleotide yields the following products on hydrolysis: 1 mol. of nicotinic acid amide, 1 mol. of adenine, 2 mols. of pentose, and 2 mols. of phosphoric acid. The pentose appears to be ribose (16, 20, 73–75), though it is possible that only one of the sugars is a pentose and the other a hexose (20). Euler and Schlenk visualize the structure as follows: (72)



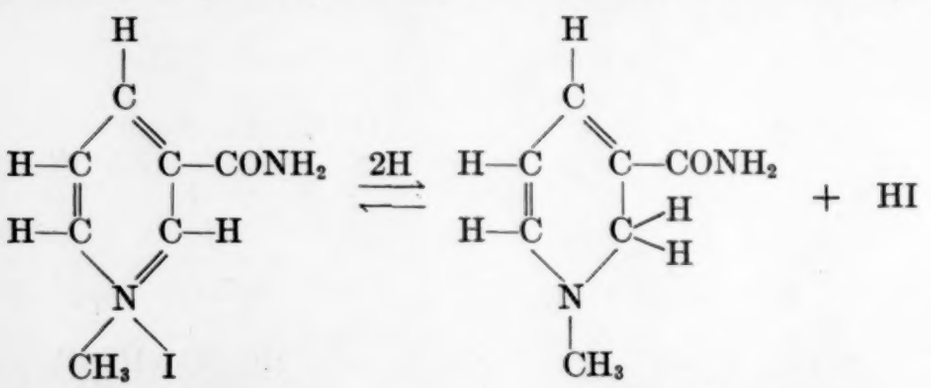
Diphosphopyridine nucleotide—coenzyme 1

The empirical formula indicates that the components are connected by the removal of 5 molecules of water. Triphosphopyridine nucleotide has a similar structure except that 3 phosphoric acid residues are present in the molecule instead of 2 (16). The 7 components are joined by the removal of 6 molecules of water. The diphosphopyridine nucleotide has been converted into triphosphopyridine nucleotide by treatment with $POCl_3$(76), or enzymatically (77–80).

The most reactive part of the molecule, both chemically and biologically, is the nicotinic acid amide residue, which readily and reversibly takes on hydrogen (16, 20). Both nucleotides are easily reduced by sodium hydrosulfite, forming the dihydrids of the coenzyme.

In alkaline solution a transient yellow color appears on reduction (65) suggestive of an intermediate stage corresponding to the semiquinones of Michaelis (81). Karrer and Benz (82) observed the transient yellow color with certain "model" compounds, and found the reaction to be characteristic for pyridines able to undergo reversible reduction. The dihydrides give off a strong white fluorescence when exposed to ultraviolet light; furthermore, they have a strong absorption band in the ultraviolet with a maximum at 340 mμ. In the oxidized form the coenzymes have neither property, which therefore can be used to identify the reduced form.

Similar spectroscopic properties have been observed in certain pyridine derivatives such as trigonellin, the methyl betaine of nicotinic acid (16, 20) or the iodo-methylate of nicotinic acid (20, 83, 84). In the presence of sodium hydrosulfite the following reaction takes place.



Nicotinic acid amide iodo methylate

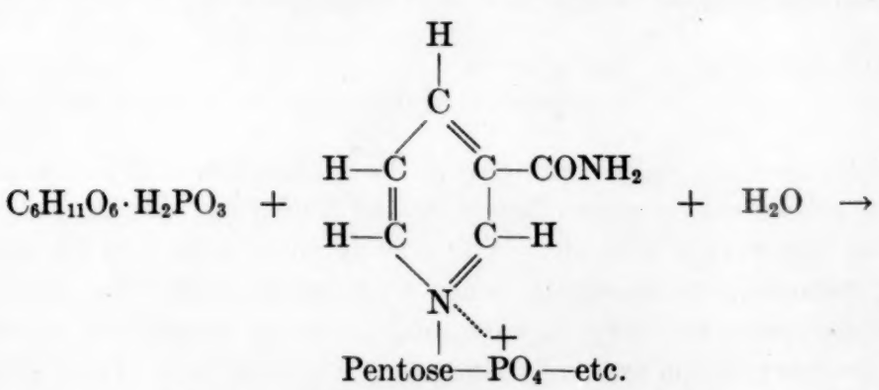
Reduced form

The double bond between the nitrogen of the ring and the α-carbon atom becomes saturated with the liberation of HI, the N changing from

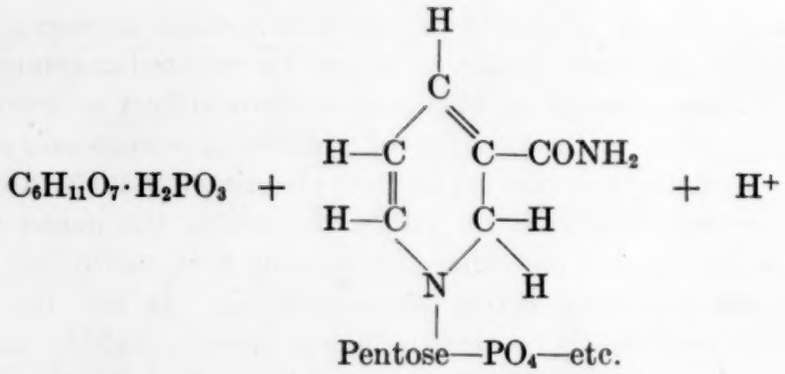
a valence of 5 to one of 3. The reduced compound fluoresces, and has an absorption spectrum similar to that of the reduced enzymes D.P.N. and T.P.N. after treatment with sodium hydrosulfite, or reduction in an enzyme system in the presence of suitable substrates and dehydrogenases. A similar reaction is therefore postulated in all 3 cases. The reduced coenzymes D.P.N. or T.P.N. as well as the model pyridine derivatives are readily re-oxidized by shaking with methylene blue, or with the yellow enzyme in the presence of air. In fact the reduced pyridines are extremely potent reducing agents, $AgNO_3$, K_3FeCN_6, and indigo-di-tri- or tetra-sulfonate being instantly reduced in the cold (85). They can even be determined quantitatively by titration with ferricyanide (84). The reduced coenzymes are stable in alkali and unstable in acid, the reverse of the oxidized form. When the coenzymes are reduced with molecular hydrogen in the presence of Pt, 6 instead of 2 atoms of hydrogen are absorbed, and the reduction is irreversible.

The mode of action of the pyridine coenzymes. The function of the pyridine nucleotides in enzyme reactions was originally elucidated for triphosphopyridine nucleotide, in a reaction by which hexose monophosphate (Robison ester) was oxidized by molecular oxygen to phosphohexonic acid. The system consisted of a specific dehydrogenase (Zwischenferment) the coenzyme TPN, and the "yellow enzyme" (flavo protein) in addition to the substrate and oxygen. The mechanism of the reaction was revealed by studying various combinations of the components of the system (15, 16, 20).

The first reaction took place when the substrate and the coenzyme were mixed in the presence of the dehydrogenase. The substrate lost two atoms of hydrogen, and the coenzyme gained two, the products of the reaction being phosphohexonic acid and reduced coenzyme.



Hexosemonophosphate + coenzyme



phosphohexonic acid + reduced coenzyme

The reaction proceeded quantitatively and in stochiometric proportions, and stopped when no more coenzyme was present to act as hydrogen acceptor.

The hydrogen was "accepted" by the pyridine ring of the coenzyme molecule in a manner exactly analogous to that already described for the "model" pyridine compounds. The double bond between the α-carbon atom and the nitrogen of the ring became saturated, one hydrogen atom adding to the carbon of the ring, the other going to form acid with the anion released as the valence of the nitrogen changed from 5 to 3. The reaction took place either in the presence or absence of air provided the three components only—substrate, coenzyme, dehydrogenase—were present. The physical phenomena characteristic of reduced coenzymes appeared, absorption band with a maximum at 340 mμ, white fluorescence under ultra violet light.

The second reaction was studied in the absence of air. To the system—substrate, coenzyme, dehydrogenase, or its reaction products—yellow enzyme (flavoprotein) was added. A reaction took place between the reduced coenzyme and the flavoprotein:

reduced coenzyme + flavoprotein
→ regenerated coenzyme + reduced flavoprotein

The hydrogen originally coming from the substrate was thus transferred to the yellow enzyme, specifically to the alloxazine ring or the flavin part of the yellow enzyme. The coenzyme returned to its original state, becoming available to react with more substrate. Since the reactions proceeded very rapidly, only a small amount of coenzyme was necessary in the system. Hydrogen was transferred from the substrate through the coenzyme, to the flavoprotein. The over-all reaction was quantitative and stochiometric, and continued until all the sub-

strate was oxidized to phosphohexonic acid, or until all the flavoprotein was reduced to the leuco form.

If air was added to the system, a reaction took place between the leuco, or reduced, flavoprotein, and molecular oxygen:

flavoprotein·2H	+	O_2	⟶	flavoprotein	+	H_2O_2
Reduced flavoprotein	+	oxygen	⟶	regenerated yellow enzyme	+	hydrogen peroxide

Thus the hydrogen originally coming from the substrate was united to molecular oxygen. The yellow enzyme was regenerated in a manner exactly analogous to the regeneration of the coenzyme. Hence in the presence of molecular O_2, only a small amount of yellow enzyme needed to be present, the over-all reaction being between the substrate and molecular oxygen. Summarizing,

$$\text{Substrate} + H_2O + \text{Coenzyme} \rightarrow \text{Coenzyme} \cdot 2H + \text{oxidized substrate}$$

$$\text{Coenzyme} \cdot 2H + \text{Flavoprotein} \rightarrow \text{Coenzyme} + \text{Flavoprotein} \cdot 2H$$

$$\text{Flavoprotein} \cdot 2H + O_2 \rightarrow \text{Flavoprotein} + H_2O_2$$

The over-all reaction is:

$$\text{Substrate} + H_2O + O_2 \rightarrow \text{Oxidized substrate} + H_2O_2$$

Both coenzyme and flavoprotein were reduced and regenerated many times, and were not used up in the reaction.

The above analysis indicates the mechanism of coenzyme action, and the general phenomenon of hydrogen transport. The reactions which take place within the living cell, however, represent a more complex extension of these basic principles. In place of flavoprotein, many compounds function in transporting hydrogen from reduced coenzyme to molecular oxygen, notably the coenzyme factor (86), diaphorase (87), cytochromes (88), and cytochrome oxidase (89). Various other possible "intermediates" have been postulated. Many substrates are broken down in the living cell, each requiring a specific dehydrogenase. In addition, there are at least two pyridine coenzymes present: diphosphopyridine nucleotide, and triphosphopyridine nucleotide.

The oxidation product of any one enzymic reaction may serve as a substrate in further oxidations. Thus the phosphohexonic acid formed in the oxidation of hexosemonophosphate by triphosphopyridine nucleotide plus the specific protein component (dehydrogenase or

zwischensubstance), is readily fermented by yeast (16). Lipmann (90) studied this breakdown of phosphohexonic acid by yeast maceration juice and found that CO_2 formation took place only in the presence of oxygen. He suggested that phosphohexonic acid might represent the primary product of carbohydrate oxidation by a path differing from the usual fermentation breakdown. Warburg and Christian (91, 92) also found that phosphohexonic acid in the presence of TPN, yeast protein, and yellow enzyme could be oxidized further. Three mols of O_2 were absorbed and three mols of CO_2 were produced per mol of hexose, hence one-half of the hexosemonophosphate was oxidized to H_2O and CO_2.

Dickens (93, 94) has studied in some detail the further oxidation of phosphohexonic acid, and finds that triphosphopyridine nucleotide takes an important part. Thus it appears that TPN not only can bring about the oxidation of hexosemonophosphate to phosphohexonic acid, but in the presence of suitable protein may carry the oxidation further.

Both diphosphopyridine nucleotide and triphosphopyridine nucleotide undergo the same type of reaction (20). Each is capable of reacting with several substrates and their specific dehydrogenases. Most enzymic systems are specific in their requirement of coenzyme; that is, they require either DPN or TPN, and one coenzyme cannot replace the other. An apparent exception is the glucose dehydrogenase system of liver (95–98) which can use either DPN or TPN. Citricodehydrase also may be able to use either coenzyme (97, 99).

The best known system requiring triphosphopyridine nucleotide exclusively is the dehydrogenase of hexose monophosphate. Many systems function only in the presence of DPN; viz., those for the dehydrogenation of lactate (11, 12, 66, 99–105), malate (26, 102, 106), glutamate (107, 108), and β-hydroxy butyrate (109). Probably the most characteristic reactions of DPN are those concerned with yeast fermentation. Cozymase (DPN) has been found to function in the enzymic dehydration of hexose diphosphate (110, 111), in the fermentation of hexose diphosphate (112, 113), and in the dehydration of triose phosphates to form phosphoglyceric acid (20, 50, 114). Furthermore cozymase is the coenzyme of alcohol dehydrogenase (57, 95, 104, 116, 117) and also functions in the mutation of aldehyde (118–121) and other compounds (114–122) in which case it may be called a "co-mutase." Certain dehydrogenase systems can be "linked" with others by means of DPN (123, 124), as for example the hydroxybutyrate-acetoacetate

and the oxaloacetate-malate systems. In the presence of the respective dehydrogenases and DPN the following reactions take place:

$$\beta \text{ hydroxybutyrate} + \text{DPN} \rightarrow \text{acetoacetate} + \text{reduced DPN}$$

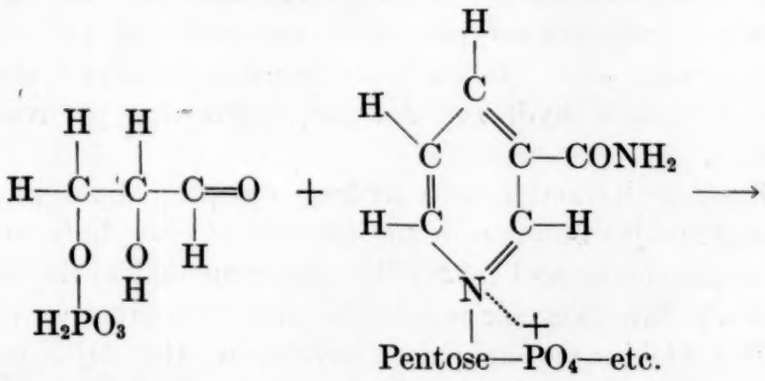
$$\text{reduced DPN} + \text{oxaloacetate} \rightarrow \text{DPN} + \text{malate}$$

the over-all reaction being:

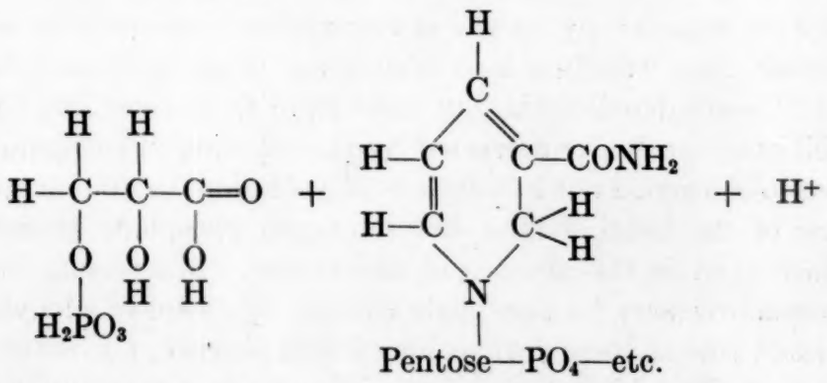
$$\beta \text{ hydroxybutyrate} + \text{oxaloacetate} \rightarrow \text{acetoacetate} + \text{malate}$$

The DPN undergoes a cycle of reduction by one dehydrogenase system and oxidation by the other.

The rôle of the coenzyme diphosphopyridine nucleotide in fermentation is perhaps best illustrated by considering a simplified form of the fermentation process. Hexose is phosphorylated to hexose diphosphate, cleaved to triose phosphate, which is then oxidized by the removal of 2 atoms of H (20, 125). Using glyceric aldehyde phosphate as a typical triose phosphate, the reaction is:



Glyceric aldehyde phosphate + Coenzyme



Glyceric acid phosphate + reduced coenzyme

The proper specific dehydrogenase must be present in addition to substrate and coenzyme (protein A in Warburg's Gär test). The reaction of the coenzyme in this stage of the fermentation process is no different than in any other dehydrogenation. However, in fermentation, the ultimate fate of the coenzyme hydrogen differs from that of hydrogen in an ordinary oxidizable system. The phosphate of glyceric acid presumably rearranges, loses phosphate and then CO_2, going to pyruvic acid, and then acetaldehyde, which acts as a hydrogen acceptor to the reduced coenzyme, the products of the reaction being ethyl alcohol and regenerated coenzyme.

For this reaction also a specific protein must be present (Warburg's protein B). This reaction, the hydrogenation of CH_3CHO, is the reverse of the dehydrogenation of ethyl alcohol for which DPN is also needed as the coenzyme (57, 95, 104, 116, 117).

The similarity between alcoholic fermentation and lactic acid formation (glycolysis) in animal tissues has long been recognized (126, 127). This similarity extends to the rôle of the coenzyme DPN in the two processes. Meyerhof and co-workers (125, 128–131) have shown that DPN acts as a hydrogen acceptor in the conversion of triose phosphate to phosphoglyceric acid. In the later stages of glycolysis, the reduced coenzyme acts as a hydrogen donator, converting pyruvic acid to lactic acid.

In addition to its action as a hydrogen carrier, diphosphopyridine nucleotide may also function in the transfer of phosphate, resembling adenylic acid in this respect (132–134). Since one-half of the diphosphopyridine nucleotide molecule is adenylic acid, this property is not surprising. Thus Ohlmeyer and Ochoa have shown that DPN is an intermediate in the transfer of phosphate from phosphopyruvic acid to glucose (135).

Phosphate transfer by means of coenzymes is remarkably sensitive to inorganic ions. Sodium ions inhibit the transfer of phosphate by either DPN or adenylic acid, but more so in the former (28, 135) and this inhibition can be counteracted by the addition of manganese. If the reaction is carried out in solutions of potassium or ammonium salts (in place of the usual sodium salt solutions) phosphate transfer will take place even in the absence of manganese. Magnesium has long been known necessary for phosphate transfer by means of adenylic acid.

The exact rôle of these various ions is still obscure, nor is the mechanism known by which diphosphopyridine nucleotide transfers phosphate. Apparently the coenzyme need not first be broken down to adenylic acid (135). It is possible that DPN takes on phosphate from

a phosphate donator, thereby changing to TPN which then passes the phosphate to a phosphate acceptor. The fact that diphosphopyridine nucleotide can be converted to triphosphopyridine nucleotide with enzymes (77, 70), microörganism (136), as well as with chemicals (76), might be considered presumptive evidence in favor of the above view, but the apparent specificity of DPN and TPN in characteristic systems suggests that these two factors are not readily interconvertible. Further study of this phase of coenzyme action will no doubt yield interesting results.

The pyridine coenzymes are essential for the growth of certain microorganisms. With Haemophilus parainfluenzae, Lwoff and Lwoff (136) have shown that "growth factor V" may be replaced by pure DPN or TPN. Adenylic acid, nicotinic acid, nicotinic acid amide, and o-dihydropropyl nicotinicamide are unable to serve as "factor V." Using the growth of H. influenzae, they have devised an extremely sensitive test for the phosphopyridine nucleotides of which they can detect 0.004 microgram. Using cultures of the bacteria grown under conditions in which "V factor" is the only limiting factor, they found that glucose, hexosemonophosphate, pyruvate, fumarate, malate, ethyl alcohol, asparagine and valine were all oxidized very slowly. Addition of either of the pyridine coenzymes brought about a rapid oxidation with all of the substrates. They found that the reaction pyridine nucleotide diphosphate $\leftrightarrows$ pyridine nucleotide triphosphate is possible within the bacteria.

The Lwoff test for the pyridine coenzymes has been applied to the blood of normal persons and pellagrins (137, 138). Kohn reports an increase in cozymase in both normal and pellagrous blood on administration of nicotinic acid (137). Spies found a decreased content of cozymase in the blood of both pellagrins and diabetics.[2] This can be corrected by giving nicotinic acid in either case, or in the diabetic, insulin also. This recalls an early controversy over the alleged identity of insulin and cozymase (139–144).

COCARBOXYLASE—THIAMIN PYROPHOSPHATE. *Nomenclature and history.* The existence of this coenzyme was first described by Auhagen (22) working in Euler's laboratory. He found that when dried bottom yeast was washed with phosphate buffer of pH 7.8 the entire coenzyme system was removed. The consequent loss of fermentation ability of the yeast could be restored by the addition of boiled yeast extract, but not by addition of a mixture of purified D.P.N. (cozymase), Mg, hexose diphosphate, and inorganic phosphate. This suggested that boiled yeast juice contained a second coenzyme. It was found that this

[2] Personal communication.

second coenzyme was a part of the carboxylase enzyme system and hence was termed cocarboxylase. Cocarboxylase had escaped previous detection because in fermentation studies, the yeast had not received the alkaline wash necessary to remove it. It was also a contaminant of impure cozymase preparations.

A relationship between cocarboxylase and vitamin B_1 was suggested by Simola (38) when he reported that the cocarboxylase content was decreased in the liver and brain of rats on a vitamin B deficient diet.

Lohmann and Schuster (145, 146) discovered the chemical composition of cocarboxylase and found that it was composed of 1 molecule of thiamin (vitamin B_1) and 2 molecules of phosphoric acid combined as the pyrophosphate. It is thus correct to refer to it as thiamin pyrophosphate, though cocarboxylase is the term in most general use.

Occurrence. Besides yeast, Auhagen (147) has reported the presence of cocarboxylase in liver, kidney, heart and blood. None was found in muscle, though recently its presence in small amounts has been reported there (148, 149). Its presence in rat brain and liver was observed by Simola (38). Tauber (150) reports the presence of cocarboxylase in a large number of vegetables. In animal tissues the largest amounts are found in liver and kidney (149). As carboxylase is widely distributed in nature, it is probable that cocarboxylase is similarly distributed. To what extent the thiamin in animal and vegetable tissues exists in the form of cocarboxylase is a disputed question. Undoubtedly the amount varies with species and with time though an increasing amount of experimental evidence suggests that cocarboxylase is predominant.

Preparation. Auhagen's first preparation (22) was that of a concentrate prepared by addition of lead acetate to dialyzed boiled yeast juice. The precipitated lead salts were decomposed by H_2S, and the filtrate fractionated by treatment with barium. A modification and extension of this preparation from boiled yeast juice was given by Auhagen (147) with which he prepared a concentrate 200 times more active than the yeast juice. The cocarboxylase was not prepared in pure form.

Lohmann and Schuster (146) prepared pure crystalline cocarboxylase from brewer's yeast. The cocarboxylase was extracted from the yeast by boiling water and precipitated from alkaline solution by barium. A mixture of dilute HNO_3 and H_2SO_4 was used to elute the coenzyme from the barium precipitate. Further purification was accomplished by precipitation with ethanol and methanol, adsorption on Frankonite KL and elution with pyridine, fractional precipitation with methanol ether, precipitation with picric acid, and then precipitation with phos-

photungstic acid and crystallization of the hydrochloride. One hundred kilograms of yeast yielded from 700 to 800 mgm. of the crystalline hydrochloride of cocarboxylase.

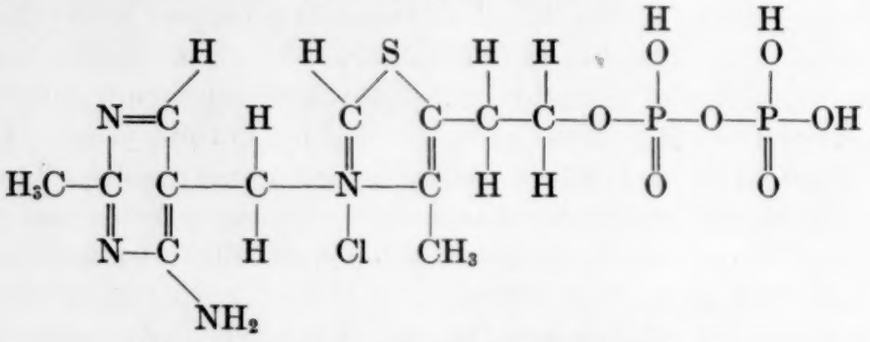
Cocarboxylase has been synthesized from thiamin by enzymatic and chemical methods. Kinnersley and Peters (151) report its synthesis by live yeast. Euler and Vestin (152) observed synthesis of cocarboxylase by the interaction of inorganic phosphate, adenosine triphosphate, and aneurin (vitamin B_1) in the presence of dried bottom yeast. Tauber (153) reported its synthesis from thiamin and orthophosphate by means of an enzyme of the duodenal mucosa of the pig. Ochoa and Peters (148) were unable to synthesize cocarboxylase with intestinal mucosa, but succeeded with liver. Stern and Hofer (154, 155) synthesized cocarboxylase by phosphorylation of vitamin B_1 with phosphorous oxychloride. Tauber (156) and Weijlard and Tauber (157) report its chemical synthesis by the interaction of thiamin hydrochloride and a mixture of $Na_2P_2O_7$ and H_3PO_4. They give a series of experiments to show that synthetic cocarboxylase as obtained from synthetic thiamin chloride is in every respect identical with cocarboxylase prepared from yeast.

Determination. The usual method of determination is that of Lohmann and Schuster (145). One gram of dried yeast is well washed with warm M/10 Na_2HPO_4 solution, and with distilled water. The residue is suspended in 10 cc. of M/10 phosphate, pH 6.2. One cubic centimeter of this washed yeast suspension and 0.4 cc. of the cocarboxylase solution are placed in the main chamber of a manometric vessel. Three tenths cubic centimeter of a sodium pyruvate solution, pH 6.2, containing 5.0 mgm. of pyruvic acid, and 0.1 mgm. of Mg, as $MgCl_2$ are placed in the side arm. After temperature equilibrium, the contents of the side arm are poured into the vessel and the cubic millimeter of CO_2 produced is observed. Sample figures taken from Lohmann and Schuster (145) are: without addition of cocarboxylase 25 cmm. of CO_2 are produced in 30 minutes, after addition of 1.0 microgram of cocarboxylase 151 cmm. of CO_2 are produced.

Westenbrink and Goudsmit (158, 159) have applied the thiochrome reaction to the determination of cocarboxylase and thiamin separately in animal tissues. The principle of the method (149) is that the free thiochrome formed by oxidation of thiamin by potassium ferricyanide in alkaline aqueous solution is extracted by isobutanol, whereas the thiochrome pyrophosphate formed from the cocarboxylase remains in the aqueous alkaline solution. Both solutions are irradiated with ultraviolet light and the intensity of fluorescence measured, from which

the concentration of thiamin and cocarboxylase are calculated. Improvements in this method have recently been made (160).

Chemistry and mode of action. Cocarboxylase is thiamin pyrophosphate; structurally it is represented as follows:



Thiamin pyrophosphate—cocarboxylase

As with inorganic pyrophosphate, one molecule of phosphate is readily removed by acid hydrolysis. The other is more resistant. Alkaline oxidation with ferricyanide produces a phosphorus-containing product with blue fluorescence in ultra violet rays (thiochrome reaction). The absorption spectrum shows 2 bands with a maximum at 245, and 260 mμ which is the same as given for aneurin (vitamin B_1 or thiamin). Cocarboxylase is an amphoteric substance. Cataphoretic experiments with the Theorell apparatus show a migration toward the cathode at a pH more acid than 3.8, and toward the anode at a pH of less acidity.

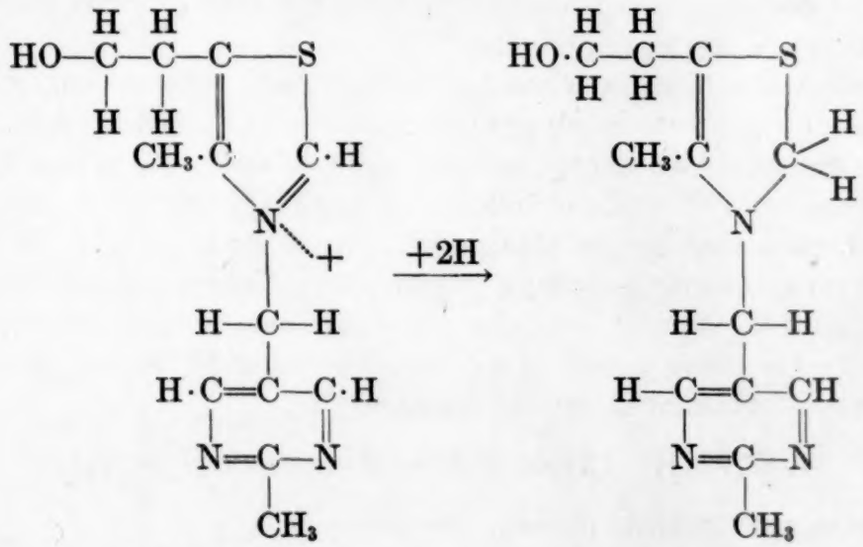
The decarboxylation of pyruvic acid in yeast by carboxylase and cocarboxylase is according to the following equation:

$$CH_3\,CO\,COOH \rightarrow CH_3\,CHO + CO_2$$

According to Neuberg and Hirsch (161) a small amount of acetoïn is produced. These products have been isolated by Lohmann and Schuster (146) from the system devised for determining cocarboxylase. They also found that acetaldehyde inhibited the enzymatic activity whereas magnesium and manganese increased the activity. Zinc and copper, on the contrary, inhibited cocarboxylase activity at higher concentrations. The optimum pH for this enzyme system is 6.3.

Lohmann's discovery that cocarboxylase contained thiamin was of great interest because it offered a definite function for vitamin B_1. A decrease in the cocarboxylase content of the tissues had previously been observed in B_1-deficient animals (38). Lohmann and Schuster (146) showed that cocarboxylase could replace vitamin B_1 in the treatment of B_1-deficient pigeons, and that it was active in the catatorulin test of Peters. Pyruvic acid accumulates in the blood and brain in

vitamin B_1 deficiency (162, 164). Disappearance of pyruvic acid and normal carbohydrate metabolism is restored upon addition of the vitamin (163–165). As cocarboxylase contains this vitamin, and definitely functions in the decarboxylation of pyruvic acid in yeast, the hypothesis was advanced that in animal tissues vitamin B_1 serves as a precursor for cocarboxylase and that it likewise functions in decarboxylating pyruvic acid. However, it has been shown (166–168) that animal tissues and certain bacteria do not decarboxylate pyruvic acid in the direct manner that yeast does, but instead oxidize it to acetic acid and CO_2, either by dismutation under anaerobic conditions (with 1 molecule being reduced to lactic acid) or directly. Lipmann (169) finds that acetone preparations of B. delbrückii can dehydrogenate pyruvic acid to acetic acid and CO_2, and yet not carry out direct decarboxylation, and regards cocarboxylase as the prosthetic group of the pyruvic acid dehydrogenating enzyme. The presence of free phosphate is necessary for the dehydrogenation; arsenate will work equally well. The dehydrogenation is independent of any dismutation. Lohmann and Schuster (146) had suggested that cocarboxylase as a prosthetic group may combine with one protein in yeast and with others in other tissues, and it may be that in Lipmann's observations it is combined with a protein component forming a pyruvic acid dehydrogenase. In this connection it is of interest to note Lipmann's observations (170, 171) on the hydrogenation of thiamin. He found that by hydrogenation with sodium hydrosulfite, one molecule of thiamin took up 2 atoms of hydrogen, and concluded that the hydrogenation takes place at the double bond adjoining the quaternary nitrogen in the thiazole part of the molecule:



Thiamin Reduced thiamin

During the reduction he observed a yellowish-green intermediate, suggestive of 2 step reduction (81). The reduction of thiamin by hydrosulfite was not reversible, inasmuch as sulfite from the $Na_2S_2O_4$ cleaves thiamin (172). In tissues, however, a reversible dehydrogenation of the thiazole ring is conceivable if the hydrogenated thiamin is stabilized (171), possibly by combination with a specific protein (dehydrogenase).

Of importance is the relationship between thiamin and cocarboxylase in animal tissues. Lipschitz, Potter and Elvehjem (173, 174) have demonstrated the simultaneous existence of both compounds in tissues and suggest that the two compounds may exist in dynamic equilibrium. They have shown that the vitamin may be phosphorylated by brewer's yeast which has been washed free of cocarboxylase with alkaline phosphate buffer, provided hexosediphosphate, vitamin B_1 and certain thermostable factors present in boiled tissue extracts are present. This synthesis was blocked by iodoacetate and it was suggested that phosphorylation occurred as a result of the esterification of inorganic phosphate coupled with the energy derived from the dismutation between triosphosphate and pyruvic acid or acetaldehyde. Ochoa and Peters (148) have objected to this work and have claimed that synthesis was not achieved by these methods. They have found that with enzyme preparations from baker's yeast there is considerable augmentation of carbon dioxide production from pyruvic acid as a substrate when vitamin B_1 is added to cocarboxylase, even in the presence of iodoacetic acid. They conclude that the increased carbon dioxide production noted in the experiments of Lipschitz et al. is due simply to activation of cocarboxylase present in the boiled tissue extract by the vitamin B_1 which is added. Apparently the discrepancy is due to the fact that "activation" of cocarboxylase is dependent upon the type of yeast used as a cocarboxylase free enzyme source.[3]

Summarizing, cocarboxylase functions in yeast with the enzyme carboxylase in decarboxylating pyruvic acid. In B. delbrückii it is a part of the system which dehydrogenates pyruvic acid. In animal tissues its precise mode of action is unknown, though it is intimately connected with thiamin and the metabolism of pyruvic acid.

COENZYME OF THE D-AMINO ACID OXIDASE. *Nomenclature and history.* An enzyme capable of oxidizing the enantiomorphs of many of the naturally occurring amino acids was discovered by Krebs (25, 175). The enzyme brought about the reaction:

$$R \cdot CH(NH_2) \cdot COOH + O \rightarrow R \cdot CO \cdot COOH + NH_3$$

[3] Lipton and Elvehjem, personal communication.

and was named d-amino acid oxidase or deaminase. Das (23, 24), working in Euler's laboratory, showed that the amino acid (proline) oxidase of Krebs requires a thermostable factor for its activity. Warburg and Christian (176) found that the d-amino acid oxidase of Krebs is a conjugated protein and consists of a protein and a coenzyme moiety, the latter being diffusible through cellophane. Together, the protein and coenzyme catalyze the oxidation of d-alanine to pyruvic acid by molecular oxygen; each alone is inactive. Straub (177), working in Keilin's laboratory, had likewise been studying this coenzyme, and found that the flavin content of his coenzyme preparations was proportional to their activity, suggesting that flavin is a part of the coenzyme. Simultaneously Warburg and Christian (178) found that flavin was a part of the coenzyme molecule. In a series of short papers (179–181) from Warburg's laboratory, culminating in a complete review of their work on this coenzyme (182) it has been shown that the coenzyme of the d-amino acid oxidase is alloxazin-adenin-dinucleotide. Other names that have been used (all by Warburg) for this coenzyme are: coferment of the d-alanine dehydrase, coferment of d-alanine oxidase, and the prosthetic group of the d-amino acid oxidase.

Occurrence. The coenzyme of the d-amino acid oxidase has been found in many animal tissues and in yeast. Straub used horse heart for the preparation. Warburg reports its presence in all tissues so far examined, namely: heart, liver, kidney, and tumor (Jensen-Sarcoma) of the rat, in addition to heart and muscle of the horse, and bakers' yeast. It may well be a ubiquitous substance. Warburg points out (182) that this coenzyme is probably associated with other enzymes, because there appears to be no parallelism between the content of the coenzyme and the d-amino acid oxidase in various tissues. For example, horse liver contains much coenzyme but no amino acid oxidase.

From $2\frac{1}{2}$ kgm. of heart muscle, Straub (177) obtained about 12 mgm. of a yellow powder very active as coenzyme. He did not report the isolation of the pure coenzyme. The muscle was minced, washed and extracted with an acetone-water mixture. After removal of an acetic acid precipitate, the coenzyme was precipitated by further addition of acetone. The precipitate was dissolved in water and reprecipitated with barium. The barium was removed and the coenzymes precipitated by adding 10–15 volumes of acetone. The yellow powder thus obtained was soluble in water, but insoluble in organic solvents.

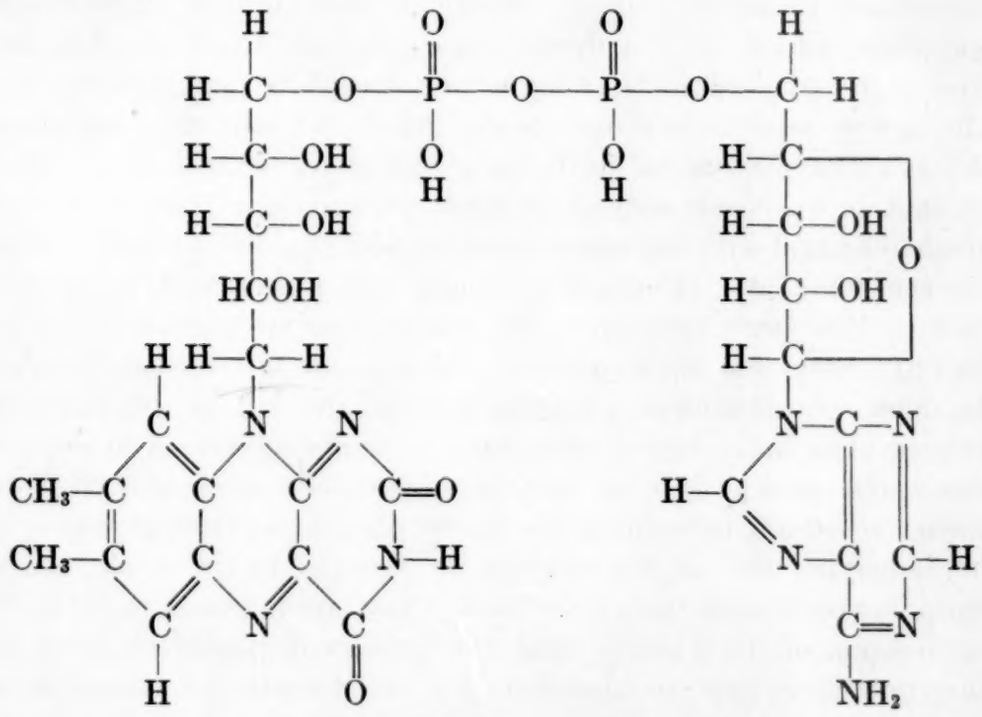
Detailed procedures for the preparation of the pure coenzyme, alloxazine-adenine-dinucleotide, from liver, kidney, and from yeast are

given by Warburg (180, 182). After extraction of the animal tissues with acetone and alcohol, the dinucleotide is extracted with water and precipitated with mercury sulfate. After removal of the mercury the dinucleotide is fractionally precipitated by silver. The silver precipitate is decomposed and the dinucleotide precipitated with barium. After removal of the barium the dinucleotide is extracted into paracresol and passed back into an aqueous solution. The pure orange colored barium salt of the dinucleotide is now carefully precipitated. From 50 kgm. of kidney or liver approximately 65 mgm. of the pure substance were obtained. A much better yield was obtained from yeast. The dinucleotide from yeast was identical with the dinucleotide prepared from kidney or liver.

Properties (182). Neutral aqueous solutions of this coenzyme retain their activity for considerable time if kept cold. The dinucleotide may be precipitated by addition of organic solvents. It may be extracted from aqueous salt solutions (NH_4SO_4 solution) by p. cresol. Normal HCl at room temperature slowly destroys the activity; at a temperature of 100° HCl destroys the activity completely in 5 minutes. N/10 H_2SO_4 or HNO_3 have little effect at room temperature. A half normal NaOH solution will completely destroy the activity in 2 hours at room temperature. When in alkaline solution, exposure to light readily inactivates the coenzyme (lumiflavin reaction); in neutral aqueous solution, exposure to light is without effect. Reversible reduction with loss of the yellow color may be accomplished with $Na_2S_2O_4$ at pH of 7.5. The dinucleotide forms insoluble salts with Hg, Ag, Pb, and Ba. The dinucleotide may be absorbed on Al_2O_3 and eluted with bicarbonate solution at pH 9.

Method of determination. The usual method of determination of this coenzyme is to determine its effectiveness in oxidizing d-alanine. An oxidation system is constructed in which the coenzyme is the limiting factor and in which its effectiveness may be observed by measuring the O_2 uptake, or by measuring the decolorization of methylene blue. For example (182) 2 cc. of a phosphate containing enzyme solution, pH 8.3 was placed in a respiration flask; d-alanine was placed in the side arm. An additional 1.34 microgram of the coenzyme was added to a second flask. Addition of the d-alanine to the first flask caused no oxygen uptake and in the second flask 22.9 cmm. of O_2 was taken up in 10 minutes. With a similar set up, Straub (177) found that in the presence of the coenzyme, methylene blue was reduced in 27 minutes, and without coenzyme it was not reduced even in 12 hours.

Chemistry and mode of action. The most significant event in the chemistry of this coenzyme was the isolation by Warburg (178) of an orange colored barium salt, which gave the absorption spectrum characteristic of flavin, and which on alkaline photolysis yielded one molecule of lumiflavin per atom of barium, and on acid hydrolysis yielded one molecule of adenine per atom of barium. Further work from Warburg's laboratory showed that the coenzyme was alloxazine-adenine-dinucleotide. Elementary analysis of the pure barium salt gave a molecular formula of $C_{27}H_{31}N_9P_2BaO_{15}$ (molecular wt.—920). Though Warburg assigned no structural formula to the coenzyme, one can visualize it as follows:



Alloxazine—adenine—dinucleotide

The absorption spectrum of this coenzyme is very similar to that of lactoflavin. Absorption bands appear with maxima at 200, 375, and 450 mμ. In the ultraviolet the dinucleotide gives a stronger absorption band. The dinucleotide absorbs slightly more than lactoflavin in the green region of the spectrum and hence its solutions appear reddish yellow as contrasted with the greenish yellow of lactoflavin solutions.

The mechanism of the action of the coenzyme of the d-amino acid oxidase is analogous to the action of the "old" yellow enzyme in Warburg's experiment with hexosemonophosphate and triphosphopyridine

nucleotide. The alloxazine ring of the coenzyme accepts hydrogen from the amino acid, and reduced coenzyme is formed. This then may be re-oxidized by molecular oxygen. Warburg has calculated the oxidative effectiveness of this coenzyme from the alanine test and finds that one molecule of dinucleotide may react with 1440 mols of oxygen per minute, which is theoretically equivalent to the oxidation of 2880 molecules of alanine to pyruvic acid.

ADENYLIC ACID. Another type of coenzyme, adenylpyrophosphate, plays an important rôle in muscle metabolism, since it functions both in anaerobic glycolysis and in aerobic respiration. The adenylpyrophosphate-adenylic acid system[4] has been extensively studied by Meyerhof, Embden, Parnas, Needham and others. Adenylpyrophosphate added to a dialyzed muscle extract transfers phosphate groups[5] to carbohydrate, forming hexose phosphates and adenylic acid. The hexose phosphates break down further, without the coöperation of this coenzyme system, until the phosphopyruvic acid stage. Here by another enzyme-coenzyme reaction, phosphate is transferred back to adenylic acid with the regeneration of adenylpyrophosphate. Thus, this coenzyme, also, is broken down and later regenerated, but in contrast to Warburg's coenzyme, the change involves phosphate, rather than hydrogen, and furthermore several steps are intermediate between the dephosphorylation of adenylpyrophosphate and its regeneration. (Hexose phosphate → triose phosphate → phosphoglyceric acid → phosphopyruvic acid.) Just as adenylpyrophosphate is considered a coenzyme necessary in forming the hexose phosphates from glycogen, so too, adenylic acid is a coenzyme for the enzymatic breakdown of phosphopyruvic acid to pyruvic acid. Two mechanisms exist for the regeneration of the adenylic acid, the transfer of phosphate from the adenylpyrophosphate to glycogen and the transfer of phosphate to creatin. Magnesium ions are essential for the action of the adenylpyrophosphate system. A detailed summary of the adenylpyrophosphate-adenylic acid system may be had from the reviews by Lohmann (185), Meyerhof (186) and Parnas and Ostern (187).

MISCELLANEOUS COENZYMES. Two other factors have been called

[4] The rôle of coenzymes in muscle metabolism has recently been reviewed by Dr. Dorothy Needham in Biokhimiya (183), but since this journal is not readily available, the essential facts regarding coenzymes in muscle metabolism are given here.

[5] Euler et al. (184) have suggested the term cophosphorylase for coenzymes of the adenylic acid type which function in the transfer of phosphate.

coenzymes, co-ferment T and coenzyme R. "Co-ferment T" which increases anaerobic glycolysis, is obtained from normal and malignant tissues (188). Its identity with pyruvic acid (189) has been questioned (190). "Coenzyme R", a factor needed by certain bacteria, was first extracted from commercial cane sugar (191), but is also present in many substances of plant and animal origin (192). It is dialyzable, heat stable, and is thought to play a part in the respiration of legume root nodule bacteria. Since there is no evidence that "coenzyme R" does actually function in respiration, it might better be classed as a growth factor until more data are accumulated.

Coenzyme activity has also been attributed to glutathione, Lohmann reporting that glutathione is the coenzyme for methyl glyoxalase (193). Apparently glutathione reacts with methyl glyoxal, and the compound formed serves as a substrate for methyl glyoxalase, the end product being lactic acid and regenerated glutathione. Methyl glyoxal, however, is no longer considered an intermediate in glycolysis, being regarded rather as an abnormal decomposition product of glyceric aldehydephosphate (194). Nevertheless methyl glyoxal offers a possible alternate path for lactic acid formation. Recently Morgan and Friedmann (195, 196) have observed that maleic acid forms addition compounds with thiol groups, and strongly inhibits the coenzyme activity of glutathione, and also of glyoxalase.

GENERAL DISCUSSION. The older concept of coenzymes was that of accessory substances assisting or speeding up reactions which were primarily determined by enzymes of the nondialyzable, heat labile, protein type. In line with the view that biological oxidation proceeds by dehydrogenation of the substrate, coenzymes were said to function in dehydrogenation by "labilizing" or "activating" substrate hydrogen, thus more readily permitting its removal by the dehydrogenase.

These older ideas have been modified. The isolation of natural compounds which function in oxido-reduction processes, and the study of artificial systems composed of these substances, have suggested their function in the living cell. Apparently intra-cellular oxidation is accomplished by means of a complicated chain of reactions in which hydrogen is transferred from the substrate through a series of compounds until eventually it unites with oxygen. Coenzymes function by actually uniting with hydrogen, and then passing it on to some other compound in the chain. Substances which are included in the line of hydrogen transfer are the dehydrogenases, the coenzymes, the coenzyme factor (86), the cytochromes (88), and cytochrome oxidase (89) (atmungs-

ferment or indophenol oxidase). Still other naturally occurring substances may function in some interlocking way with this chain of dehydrogenation, or perhaps offer alternate routes of transfer from substrate to oxygen, for example, yellow enzyme, certain of the dicarboxylic acids (197, 198), glutathione (199), and adrenaline (200). In fact, any naturally occurring substance that is readily and reversibly oxidized and reduced in the cell can be suspected of functioning in intracellular metabolism. Considerable variation is possible in the routes of hydrogen transfer postulated in various tissues and in the tissues of various species, but that such routes do exist appears to be generally accepted.

Coenzymes are no longer looked upon as accelerators[6] in any supplementary sense, but rather as essential, integral members of a complicated "bucket brigade," transferring hydrogen or phosphate from compound to compound; they empty their buckets and come back for more. They are "carriers" of both hydrogen and phosphate.

But protein enzymes of the traditional type, the dehydrogenases, must be present to bring about the transfer of hydrogen from substrate to coenzyme. The coöperation between enzyme and coenzyme is obviously as real today as when the word "coenzyme" was first coined, but the demonstration of hydrogen transfer, however significant, does not clarify the relationship between enzyme and coenzyme. Two points of view have been expressed. The first is that the dehydrogenase and the coenzyme, though related in function, are separate entities, and uncombined. The second is that the coenzyme unites with the protein (dehydrogenase) to form a conjugated protein, or "proteid," of which the coenzyme is the prosthetic group.

As evidence for the former view the great specificity of the dehydrogenases has been stressed. Some are specific to only one compound, others, to a class of compounds. Thus, lactic dehydrogenase acts only with lactic acid, glucose dehydrogenase with glucose, xanthine dehydrogenase with xanthine and simple aldehydes, α-glycerophosphate dehydrogenase with α-glycerophosphate, etc. The oxidation of any of these substrates takes place only in the presence of the proper dehydrogenase and a suitable hydrogen acceptor.

The action of certain specific dehydrogenases can be inhibited by

[6] Loevenhart (6) was the first to attempt a limitation of the term coenzyme. He suggested that the term "be reserved for those substances which are absolutely necessary in order that the enzyme manifest itself at all," and that substances which merely accelerate enzyme processes may be termed accelerators.

substances closely related to the substrate, as for example, malonic acid, which inhibits succinic acid dehydrogenase (20). This inhibition is explained on the basis of a combination of the dehydrogenase and the inhibitor—"tying it up." More direct evidence for the union of dehydrogenase and substrate has been obtained by cataphoresis experiments. From an analysis of the rates of migration of mixtures of hexose monophosphate, triphosphopyridine nucleotide, and the dehydrogenase in an electric field Theorell (202) concluded that the substrate formed a compound with the dehydrogenase, and also with the coenzyme. No evidence of an enzyme-coenzyme union was observed. Enzymic oxidation, therefore, might be viewed as follows:

(1) substrate + dehydrogenase → substrate-dehydrogenase complex with "activated" hydrogen

(2) substrate-dehydrogenase complex + coenzyme → oxidized substrate + dehydrogenase + reduced coenzyme.

Warburg holds a different view (203). He emphasizes the combination of protein (dehydrogenase) and coenzyme. In a sense this is a modern adaptation of the Willstaetter idea of a "colloidal carrier" and a non-colloidal "active group," but whereas the older workers attached no particular significance to the protein "carrier," Warburg, of course, appreciates its specificity. The analogy to hemoglobin has been used—the protein (dehydrogenase) corresponding to the globin, and the coenzyme to the hematin, the combination being necessary for activity. As there are many different globins in various animal species combined with the same hematin, so there can be many protein dehydrogenases combined with the same coenzyme.

Warburg's nomenclature differs from that generally used in English. The dehydrogenase of hexose monophosphate is called "zwischensubstanz," the combination of protein and coenzyme is called "enzyme."

Protein component + coenzyme → hydrogen transporting enzyme
(Zwischensubstanz)
(Dehydrogenase)

This point of view most certainly applies to the flavin enzymes. Thus Theorell (204) succeeded in splitting the yellow enzyme into a protein component and a prosthetic group by dialyzing a solution of the yellow enzyme against 0.02 N HCl for 72 hours. The prosthetic group, flavin phosphate, was dialyzed away leaving the undenatured protein component. This protein could be combined with flavin phosphate pre-

pared synthetically (205) or by methyl alcohol extraction (204), to re-synthesize an active yellow enzyme. Nonphosphorylated flavin did not form a compound with the protein of the yellow enzyme.

d-Amino oxidase, likewise, has been cleaved into a protein component and an active prosthetic group by hydrolysis with dilute HCl (182). An $(NH_4)_2SO_4$ solution of the enzyme was used. The prosthetic group was found to be the alloxazine-adenine-dinucleotide, and corresponds to the flavin-containing coenzyme previously reported (176, 177). Protein and prosthetic groups could be recombined to give the active "enzyme."

TABLE 1

	Protein component (dehydrogenase) (zwischensubstanz)	*Coenzyme* (prosthetic group) (active group)	*Enzyme* (Enzyme-complex)
1	Protein (O_2—reduced coenzyme)*	+ alloxazine nucleotide	Alloxazine-proteid† (O_2—reduced coenzyme)
2	Protein (O_2—reduced coenzyme)	+ alloxazine adenine dinucleotide	Alloxazine adenine dinucleotide proteid (O_2—reduced coenzyme)
3	Protein (methylene blue—reduced coenzyme)	+ alloxazine adenine dinucleotide	Alloxazine adenine dinucleotide proteid (methylene blue—reduced coenzyme)
4	Protein (O_2—d-amino acid)	+ alloxazine adenine dinucleotide	Alloxazine adenine dinucleotide proteid (O_2—amino acid)
5	Protein (O_2—xanthine)	+ alloxazine adenine dinucleotide	Alloxazine adenine dinucleotide proteid (O_2—xanthine)

* The subscripts in parenthesis indicate the oxidizing agent and the substances oxidized.
† Warburg uses the term "proteid" to refer to the union between the protein and prosthetic group.

A new synthetic enzyme was prepared by adding alloxazine-adenine-dinucleotide (prosthetic group or coenzyme from d-amino oxidase) to the protein component of the "old" yellow enzyme (206). The resulting compound transferred hydrogen from dihydropyridine to molecular oxygen very much as did the old yellow enzyme.

Still another flavin enzyme has been isolated by Haas (207), working in Warburg's laboratory. The enzyme was found in yeast, and also contains the alloxazine-adenine-dinucleotide as prosthetic group, but the protein component differs from that of the enzymes previously discussed. The enzyme will oxidize reduced TPN, but reacts much more rapidly with methylene blue than with molecular oxygen.

Ball, working in Warburg's laboratory, reports that xanthine oxidase, also, may be split into a protein component and a prosthetic group (208, 206). Here too the prosthetic group is alloxazine-adenine-dinucleotide, the same as the coenzyme of the amino acid oxidase and of the new yellow enzyme. As yet it has not been possible to regenerate an active xanthine oxidase.

These observations may best be summarized by the accompanying table (table 1) taken essentially from Warburg and Christian (206).

In 1 and 2 the protein component is the same and the coenzyme (prosthetic group) is different. In 2, 3, 4 and 5 the protein component differs and the coenzyme is the same. It is the protein component that gives the enzyme specificity and determines whether the coenzyme shall accept hydrogen from xanthine, from amino acids, or from the reduced phosphopyridine nucleotides.

There is, therefore, ample evidence that the alloxazine-adenine-dinucleotide combines with specific proteins to form active "enzymes." It is merely a question of nomenclature whether the protein component alone shall be called "enzyme," or whether the protein-coenzyme complex, the proteid, is called the "enzyme." Both components are capable of uniting with one another to form stable compounds. Xanthine oxidase, the classical example of an enzyme which does not require a coenzyme, is one of these stable proteids. The reason it did not appear to need a coenzyme was that the coenzyme (prosthetic group) was already present, firmly attached to the protein component. When the proper chemical procedure was found, it was possible to separate the prosthetic group. Future work may show that other enzymes, which today are considered acting without coenzymes, may be dissociated by the proper chemical treatment into a protein component and an active prosthetic group having the properties of a coenzyme.

It is quite possible that the pyridine nucleotides, also, combine with specific proteins to form an active complex. However, the resulting pyridine proteids are much more easily dissociated than the alloxazine proteids; and hence the prosthetic group (or coenzyme) can be removed more readily (209–212). Dissociation constants have been determined for the proteids of both the reduced and oxidized form of the pyridine-nucleotides, and the reduced form of the coenzyme found to be much less firmly bound than the oxidized form (211).

The enzyme-coenzyme relationship might be pictured as follows: reaction between coenzyme and substrate takes place when the proper dehydrogenase, a large molecule, seizes the coenzyme with one hand, the substrate with the other, and bumps the two together. The co-

enzyme takes hydrogen from the substrate, and the reduced coenzyme breaks away. It can then pass its hydrogen on to a suitable acceptor, either as free reduced coenzyme or in the presence of some other specific protein.

Recently Corron and Green (213) have isolated a flavo-protein from milk which markedly accelerates the oxidation of reduced DPN by methylene blue or pyocyanine. The prosthetic flavin appears to be a phosphorylated derivative, not identical with lactoflavin phosphate. It can function as the coenzyme of d-amino acid oxidase, but the authors claim that its chemical and physical properties are not identical with those of the amino acid oxidase coenzyme. They found that the milk flavo-protein does not itself become reduced during its catalysis of hydrogen transfer, and assume that it forms a complex with reduced coenzyme which accelerates the reaction with methylene blue.

The relationship between coenzymes and vitamins is particularly interesting. In both diphosphopyridine nucleotide and triphosphopyridine nucleotide the enzymatically-active group is nicotinic acid amide, which is also active as a vitamin in the cure of canine black tongue (214) and human pellagra (215). In cocarboxylase the active group is thiamin or vitamin B_1. In the coenzyme of d-amino acid oxidase the active group contains riboflavin, a member of the vitamin B_2 complex (216, 217). Thus, in addition to clarifying our knowledge of cellular metabolism, the study of coenzymes has materially enhanced our knowledge of the vitamins.

It is of interest that all of the coenzymes discussed in this review contain phosphate. Many biological reactions, particularly those involving marked energy changes, are intimately dependent upon phosphate containing compounds and frequently involve phosphate transfer. Reactions involving coenzymes are no doubt important sources of energy to the living cell. Likewise it is interesting to speculate on the fact that coenzymes are nucleotides,[7] a class of compounds characteristic of nucleoproteins and the cell nucleus. Cytologists have long maintained that the nucleus is the controlling, physiologically dominant part of the cell. Geneticists have placed the dominance of the nuclear material on the chromosomes and their genes. The demonstration of the rôle of certain coenzymes, nucleotides, in biological oxido-reductions in an interesting biochemical counterpart of the cytological interpretation of nuclear function.

[7] Though cocarboxylase as isolated in the laboratory is not a nucleotide, one can readily postulate that within the cell it exists in a "nucleotide-like" combination.

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DENTAL CARIES

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A previous discussion of dental caries (Marshall, 1924) compared some of the historical viewpoints with the modern theories of its etiology. During the fourteen years which have elapsed since the publication of the review, a number of contributions have served to advance, in some degree, the ideas previously mentioned. By assessing arbitrary values to these later reports it is hoped that progress in research dealing with caries may be interpreted in terms of etiology, pathogenesis and prevention.

The original classification of the etiological factors followed for convenience an alphabetical arrangement, namely: A, anatomical; B, bacteriological; C, chemical; D, dietary; E, endocrine; F, failure in mouth hygiene; H, heredity. It will be noted that the first three, A, B, C, are essentially local in effect; the next two, D and E, are of systemic importance and the last two, F and H, comprise both local and systemic causes. The terms "direct and indirect," when applied to a classification of etiological factors in caries, have little meaning, for the reason that the relative importance of the seven sub-heads varies with age, environmental vicissitudes, habits, health and probably other, as yet undetermined, agents. Accordingly, each heading again will be discussed and the previous conclusions compared with current hypotheses. Since each factor has been intensively studied in many laboratories the viewpoint today has brought into sharper focus certain aspects which previously have been obscure.

It is a well known clinical observation that dental caries develops much faster in the deciduous teeth than in the second set. This inequality is due in part to dissimilarities in degree of calcification, and in the size and position of the teeth. Even slight irregularities of enamel surface favor plaque development and thus become susceptible areas for a primary carious lesion.

One of the greatest advances in the study of the pathogenesis of caries has been the experimental production of hypoplastic teeth. Areas which are deficient in formation not only are less dense but show also

surface inequalities or pits. The greater part of the research dealing with malnutrition, whether of dietary insufficiency, unbalanced mineral intake or endocrine dysfunction, is devoted to the study of the factors adversely affecting dental development.

Many writers, including Williams (1897), Walkoff (1915), Mummery (1923), and Marshall (1936), have confirmed the clinical and laboratory observation that there is scarcely a tooth in man or lower animal which does not present areas of incomplete formation. Dental literature contains many papers dealing with dental hypoplasia from the standpoint of pathological anatomy. However when a "defect" is so widespread, it ceases to have any diagnostic significance; on the contrary it becomes of histologic, rather than histopathologic interest. An individual six feet in height is as normal anatomically, as one who is five feet five inches in height; but at seven feet, or three feet in height, one postulates abnormality. So with dental tissues. But when an area of incomplete formation becomes a punctate depression, easily discernible with the unaided eye, or when deficiencies in cement substance impart an altered translucency to the enamel rods, either as white spots or as mottling, or when unclosed pits and fissures on incisal and occlusal surfaces are wide and deep enough to be recognized clinically, these defects present pathogenic significance.

One of the fundamental observations upon which a great deal of the subsequent research was built was a physico-chemical study conducted by Entin (1929). He suggested first, that caries might be regarded as a symptom of latent avitaminosis D; and second, that enamel and dentine possess properties of a semi-permeable membrane toward solutions of salts and dyes. Thus hypoplastic teeth are more permeable than "normal" ones in which calcification is dense and nearly completed. In an effort to increase calcium deposition in tooth structure Fish (1935) fed maximum doses of vitamin D plus calcium carbonate but found no measurable change in the calcium content of formed dentine in erupted teeth. As was emphasized in an earlier communication (Fish, 1932), caries begins in a permeable crevice or fault area. Upon the eruption of the tooth, the "fault" becomes filled with oral fluids containing food particles and microörganisms. If the lesion progresses slowly, a slight irritation arises in the dentine beneath the carious area. Hypercalcification results and thus a barrier is formed against the further invasion by caries. Beust (1931) confirmed Fish's views when he stated that the resistance which a tooth offers to caries is directly proportional to its hardness. The pathogenesis of caries

depends not only upon defective dental structures but also upon the susceptibility of the patient to the organisms of caries. But the questions of seasonal variations (Erpf, 1938) and of control of susceptibility remain unanswered, nor can the probable hereditary influences be assessed. In short, racial and hereditary factors require extensive study.

Karshan, Weiner, and Stofsky (1934), using specially prepared aqueous extracts of enamel and dentine, were unable to demonstrate important differences in carious and non-carious teeth in respect to hydrogen ion concentration, calcium, phosphorus and carbon dioxide. Experiments were made also on the determination of soluble protein and it was found that although the enamel presented only slight changes in carious and non-carious teeth, the dentine showed quite significant variations.

Applebaum and Adams (1938) report interesting data obtained from the use of the Grenz ray in the histopathologic study of carious teeth from rats. The several types of caries have been classified first, into those lesions found in fissures; second, in fractured enamel of molar cusps, and third, in fractures on the tip of the cusp. The Grenz ray brings out very clearly that fractures are adventitious, occurring both in carious and non-carious teeth and therefore not a part of the pathogenesis of caries. Decalcification is the important event. The evidence thus refutes the idea that in the rat caries develops only when the enamel is fractured.

Hodge and McKay (1933) by developing a new technic, have presented data which indicate that enamel comprising the outer third is greater in micro-hardness (Bierbaum hardness number) than that of the middle or inner thirds. This fact would have even greater significance if the teeth which were tested had been extracted from patients of known age.

Additional histological studies are offered by Cape and Kitchin (1930). Confirming Orban's views upon the eccentric deposition of lime salts in human enamel, they reported histologic observations made by studying sections illuminated with polarized light and with crossed nicol prisms. This field of research offers fascinating opportunities for further study, particularly of the different types of caries, both clinical and experimental.

Chemical determinations of "trace elements" in human teeth have identified strontium, barium, silver, lead, chromium, tin, and zinc and possibly traces of magnesium (Lowater and Murray, 1937). Although

teeth from individuals residing in and around London contain doubtful traces of fluorin, those from Maldon, using water having a relatively high percentage of fluorides, show the definite chemical reactions of these compounds. When fluorides are added to rats' diets these halogen derivatives are demonstrable in enamel, and such tissues may be classified as hypoplastic (Smith and Lantz, 1932). Mellanby's experiments (1928, 1930, 1932a, 1932b; Mellanby and Pattison, 1932) although criticized from other aspects (Shaw, 1932; Fish, 1932; Marshall, 1933) are acceptable at least in this connection, that defective dental calcification appears in teeth of experimental animals when diets are fed which are deficient in vitamin D and calcium and phosphorus.

In South Africa, as noted by Shaw (1932), rickets is practically unknown and if Mellanby's views are correct caries should be correspondingly low. But the opposite is true. In an examination of about 600 children only 6.6 per cent showed clinical evidence of sound teeth and 93.35 per cent presented one or more carious areas, the average for all being 4.84 carious teeth each.

In spite of these divergent views there is justification for the statement that of all the factors entering into the etiology of caries the imperviousness, or lack of it, of dental structures to certain solutions appears to be extremely important. Perhaps it was this thought that prompted Fish (1932) to define caries as a "saprophytic phenomenon occurring either in morphological crevices of development or acquired faults in the tooth surface" and to say that "the tooth is powerless to prevent the onset of caries though the progress of the disease may be retarded by vital reactions in the dentine."

Enright and collaborators (1932, 1934; Enright and Friesell, 1933; Enright, Friesell and Trescher, 1932) offer additional confirmation. They have summarized their experimental data by indicating that deficient diets and ensuing defective nutrition result in formation of teeth more susceptible to acids of fermentation than fully calcified teeth.

Comparative studies (Hess, Abramson and Lewis, 1934) on the incidence of caries in rachitic and non-rachitic children have shown that in rickets, the incidence in deciduous molars is 27.4 per cent, and is greater than that found in non-rachitic patients. It is suggested that the interference in calcium metabolism occurring in rickets is of greater etiological importance in caries of deciduous teeth than in caries of permanent teeth. This has been denied by Shelling and Anderson (1936), but confirmed by Eliot et al. (1934), who agree on several points with the group of research workers in Hess' laboratory, first, that

dental caries is found more often in children with a known history of rickets; second, that caries occurs more often in hypoplastic teeth; and third, that anti-rachitic therapy in infancy reduces the incidence of enamel hypoplasia.

From time to time there appear in the literature statements to the effect that the incidence of caries is higher in girls than in boys. Klein and Palmer (1938) conducted a survey on 4,416 children of school age, which indicated that caries incidence of girls very closely parallels that of boys. This would tend to support the findings of Cunningham (1934) at least in this respect, that disturbances of calcium metabolism incident to menstrual periods have little etiological effect in caries. In Cunningham's extensive survey physical examinations and dental charts of 11,000 women students were studied. She failed to find any significant relation between the number of damaged teeth and histories of illness, or menstrual function, or physical measurements. The count of damaged teeth, unfortunately, did not include the number of extracted teeth, an oversight which the author herself mentions in her report. Perhaps the admission of these figures would have led to a different summary of the research.

Another field for investigative effort, suggested by Bichlmayer (1935), is concerned with the badly broken down and carious tooth as a portal of entry for diseases such as tuberculosis. This matter has also received the attention of Snow (unpublished report) in his observations at Weimar Sanitarium. Although the possibility that infection may be carried through this channel cannot be denied, the preliminary data are too scattered to permit further statements.

To formulate an hypothesis which will reconcile the diverse opinions on the anatomical factors in the etiology of caries which have been so freely expressed, would be a task too laborious for this short review. An analysis of the facts however is possible. It becomes increasingly evident that structural weaknesses in enamel and dentine occur so frequently and have been produced so easily experimentally, that the relative density of teeth may be the controlling factor in immunity and susceptibility to caries.

The former concept of hypoplasia not only is inadequate but in certain respects is wrong. Attention is first invited to the dictum of G. V. Black and others to the effect that a tooth once erupted does not change in degree of calcification. Quite the contrary is the case. Both enamel and dentine in older people are much more difficult to cut with burr, stone or chisel than in young adults. The differences are even

greater between human permanent and deciduous teeth, and are less noticeable in similar teeth from monkey or dog. Dental density in humans usually increases with age. In the event of disease, either infectious or metabolic, this condensation of hard tissues may not develop.

Of equal importance is the next fact, now established in many laboratories, that both enamel and dentine possess properties of permeability provided that the dental pulp retains its vitality and is neither infected from without or within, nor inflamed from any mild but persistent trauma (severe trauma causes pulp necrosis). The passage of substances through these tissues is controlled by the density or degree of calcification in each tooth; the less dense the greater the permeability. If the mineral intake is unbalanced or if the nutritional factors become inadequate at any period from fetal life to twenty years of age, or if the individual contracts an exanthem, any or all of these circumstances are reflected in the degree of dental hypoplasia so produced (Marshall and Lucia, 1938). Dental permeability is greater when the nutritional factors are inadequate; thus not only are defectively calcified teeth more liable to be infected with caries but erupted teeth also change. It is possible that erupted teeth, instead of developing the usual condensation phenomena, retain their degree of permeability incident to their eruption, or, by a reverse reaction, become more permeable. Thus the inherent defense mechanism fails and rampant caries develops. Nor is this frank speculation, but is based, as will be shown later, on clinical and laboratory data.

The older idea, that the exanthemata of childhood were mainly responsible for the occurrence of hypoplastic teeth must be discarded. The knowledge of nutrition which has grown so rapidly during the last twenty years develops the premise that dental hypoplasia is a sequel, not only of certain infectious diseases but also of metabolic diseases in which category occur dietary deficiencies and endocrine dysfunctions. These facts also will be more fully developed in succeeding sections. Indeed hypoplasia of teeth can no longer be regarded as a disease; it is found so frequently that it must be classified as a minor anatomical variation.

Although the interest in the bacteriology of caries has been as keen as in its histo-pathology, the contributions have been less spectacular, but none the less important. Confirmation of the theories of W. D. Miller on the subject seem always to be the starting point, and in some instances unfortunately, the end point as well.

A relief from this reiteration is to be found in a paper by Dobbs (1932). In his research upon mucin plaques, it was demonstrated that these accumulations are more permeable to dextrose solutions than to salivary buffers. Carbohydrates in the mouth are hydrolyzed to various organic acids such as lactic acid, which may accumulate in concentrations great enough to decalcify enamel. It is at this point that Fosdick and Hansen (1936), and Fosdick, Hansen, and Messinger (1937) considered the possibility that acids, other than lactic, are produced as degradation products of carbohydrates. They suggest that pyruvic acid, although it has never been isolated as such from human saliva, is actively concerned in dental decalcification. However, the reductase activity of various oral flora transforms pyruvic acid to lactic acid in the presence of insoluble calcium salts.

Further research by Hansen, Fosdick, and Epple (1937) upon the formation of reducing sugars from soluble starch, has extended and confirmed their preliminary studies. In the initial stages of caries *B. acidophilus* by itself is incapable of exerting any decalcifying action on powdered enamel. This is the point which is at variance with Miller's theory. However, both in the initial and in the well developed lesion, when *B. acidophilus* is grown in symbiosis with *B. aerogenes* or with *Saccharomyces cerevisiae*, decalcification proceeds readily. This explains the observations of Mull, Bill and Kinny (1934), who have stated that *B. acidophilus* was not consistently present in caries nor consistently absent in immunity.

Bibby (1935), Bibby and Sedwick (1933) confirmed the theories of early experimenters, for example, Bodecker (1927, 1934), Bodecker and Applebaum (1932), Williams (1897) and others even earlier, who proved that decalcification of enamel is the initial process in caries rather than proteolysis of organic matrix of this tissue. This point had been questioned and needed re-investigation.

Jay and his colleagues (see bibliography, 1929, 1930, 1932, 1933, 1936, 1938) summarized the studies of the Michigan group by stating that:

1. Dental caries is unrelated to variation in dietary constituents nor can it be arrested by adding mineral and vitamin preparations. A diagnostic relationship exists however between oral lacto-bacilli and the amount of carbohydrate in the diet; thus, by restricting carbohydrate in diet, rampant caries can be effectively treated. One might be forced to quibble a bit in an effort to adjust the first statement with the latter; in one instance caries is unrelated to diet, and in the second, it can be controlled by diet. However, the inference is that dental

caries is unrelated to variation in dietary constituents except carbohydrates which, when restricted, play indirectly a part in reducing bacterial activity and consequently caries development. W. D. Miller said something to this effect in 1893.

The conclusions of the Michigan group lack confirmation in the experiments of Tucker (1932). He reports that aciduric streptococci were found in mouths of all children irrespective of the presence of caries. Furthermore, the ingestion of citrus fruits did not affect the bacterial count. The research indicates that *L. acidophilus* bears some relationship to carious cavities but is not an obligate factor in etiology. Boyd, Zentmire, and Drain (1933) confirm the observations of Tucker and extend them by concluding that children on a supervised dietary regimen exhibit no characteristic difference in the morphological nature of oral flora, from those whose diet is unrestricted. Furthermore Rosebury and Karshan (1931) report that a diet high in carbohydrates, but lacking entirely, or containing less than the physiological requirement in calcium and vitamins C and D, failed to produce caries, even though oral lactobacilli were fed in an adhesive gum tragacanth paste. But Etchells and Devereux (1933) were successful in an experiment in which pure cultures of aciduric organisms were introduced into a sterilized coarse ration. Control animals failed to develop caries, all others showed extensive lesions. Of course the experiments are not parallel except in so far as the bacteriological aspect is concerned, but on that point, at least, the success of Etchells and Devereux is convincing.

Hollander (1934), reporting for the Columbia University research group, concludes that the process of dental decay is associated with environmental factors, such as food retention and lacto-bacillary fermentation, and with physiological factors related to calcium and phosphorus metabolism. This summary confirmed previous reports from Columbia and other institutions.

Johnston et al. (1933, 1936) compared oral flora in rats with and without caries-like lesions and concluded that *Lacto-bacillus acidophilus* is not associated with the incidence of rat caries; in fact, this type of organism is normally present in all cases. In children a low count of oral bacteria was found alike in carious and non-carious mouths. Although they attach less importance to the presence of lacto-bacilli as an etiological factor of primary importance in caries, they suggest that teeth may develop a lowered resistance arising from nutritional disturbances and thus are more readily attacked by bacteria. This again infers that dental hypoplasia is a predisposing factor.

Rodriguez (1931) found that a high bacterial count of colonies per cc. of saliva apparently bears some relationship to age incidence of caries. Between 10 and 14 years there is a gradual rise, but the succeeding four years (14 to 18) show a slight decrease. In this connection Lyons (1935) studied cultures from carious teeth and presented data upon the percentage distribution of *L. acidophilus* in accordance with the age of the patient. During the first ten years this organism occurred in 84 per cent of the cases, and dropped to 66 per cent between 10 to 20 years; 49 per cent between 20 to 30 years; 20 per cent between 30 to 40 years; 14 per cent between 40 to 50 years; and 2 per cent from 51 on. Furthermore with advancing years there occurred a change in the type of organisms, in which flagellated bacilli and streptococci ultimately predominated. Rat caries was produced by mixing selected cultures of acid-forming bacteria with food.

In summarizing this section on the bacteriology of caries it is evident first, that both laboratory and clinical data suggest opposing points of view. One group maintains that the type of oral flora is the same in carious and non-carious mouths. The other group is equally emphatic in pointing out that aciduric organisms predominate in caries. It is possible that the suggestions of Hansen, Fosdick and Epple (1937) may serve to reconcile these two theories.

Although the chemical changes in saliva which were discussed in the earlier review have received scant attention, a few investigators have developed this field of research. Forbes (1931) in an excellent paper dealing with the theoretical aspects of the etiological factors, says that the neutralizing power of saliva against localized acid accumulations becomes a very important agent in the prevention of caries. It is likely that the concentration of alkaline salivary buffers is primarily determined by the alkali reserve of the blood. Since this in turn is partly a dietary problem it is necessary to differentiate between a diet that is potentially and one that is actually alkaline. It has been pointed out by Cole, Speer and Heyle (1929) that calcium and phosphorus retention is favored by a diet potentially alkaline; but on the other hand, retention is decreased when the diet is actually alkaline. One pauses to speculate upon the effect on the population of the promiscuous and unsupervised use of alkaline salts, which may increase actual alkalinity beyond the physiological optimum, and thus become one of the most insidious forms of self-medication. By following a diet which is potentially, rather than actually alkaline in character, blood alkali reserves

may be maintained. Recalling the statements of Forbes that the alkali reserves of the blood control the alkali reserve (neutralizing value) of saliva, it is evident that dietary supervision is a necessary part of the prophylaxis of caries.

The work of Eddy (1933, 1934) and Eddy, Heft, Rosenstock and Ralston (1933) dovetails very nicely with reports from other laboratories. They emphasized the fact that up to that time (1933) no worthwhile evidence had been presented to prove that sugar favors a constant production of lactic acid-forming organisms. The deleterious effect of sugar is found first, in the lowering of the blood phosphorus; second, in raising blood pH without changing the blood calcium; thus supporting the unpublished observations of Klein, Kruse and McCollum. The decrease in blood phosphorus results in a corresponding temporary decrease in salivary phosphorus. Therefore, salivary phosphorus can be controlled to some extent by dietary supervision. For example, it is lowered by rapid ingestion of sugar or other less readily digested carbohydrates, or proteins; it is unaffected by fats; and may be temporarily increased by inorganic phosphate or by dietary substances containing this element in correct nutritional proportions.

Determinations of hydrogen ion concentration still continue to interest some laboratories. However, there has never been a research described with sufficient accuracy in which the results have been other than negative, in so far as this characteristic of saliva is concerned (Eddy, 1934). Alteration of pH neither indicates immunity from, nor susceptibility to, caries; the variations are too small. Karshan (1931) and Krasnow (1936, 1938) reported the pH of blood and saliva, and determinations of calcium and phosphorus, protein, and carbon dioxide. They have pointed out that titratable alkalinity is a better criterion than the hydrogen ion concentration, because the pH values observed in caries-immune and caries-susceptible individuals either actually overlap or else differ so slightly as to be without significance, both from the diagnostic and etiologic standpoints. Perhaps other interpretations would be found if greater dilutions of standardized solutions were made. It has been shown that the one one-hundredth normal solutions give less accurate results than two-hundredth normal. The choice of indicators is equally important. Para-nitro-phenol far surpasses methyl orange for the determination of alkalinity; and phenophthalein titrated against sodium hydroxide two-hundredth normal is a very satisfactory method for determining acidity of saliva samples (Marshall, 1917, 1934).

It is suggested by Schnack (1932) that in considering the etiology of caries from the standpoint of the importance of saliva, it is necessary to divide the topic into three parts; dental caries is favored first, when the buffer action of the saliva is diminished; second, when imperceptible acid regurgitations occur from the stomach; third, by acid fermentation in localized areas around the teeth. White and Bunting (1935, 1936) tested findings reported by Grove and Grove (1935) and were unable to confirm the statements that the activity of dental caries is correlated with ammonia content and pH of saliva. Champion (1932) by producing a temporary alkalosis and later an acidosis by ingestion of different buffer salts, altered the carbonate-bicarbonate ratio of the blood and the pH of the urine. The saliva was unchanged.

Mull, Bill and Kinny (1934) determined titratable acidity in salivas of pregnant women both carious and immune, and showed that the differences were too slight to be of any diagnostic significance. If total neutralizing values had been computed these additional data might have been of considerable interest. Neither were they able to show that the values of calcium and inorganic phosphorus in pregnancy indicate any relation to the condition of the teeth. Hubbell (1933) and Hubbell and Bunting (1932) report similar results for calcium and phosphorus.

Boyd (1933, 1937) and Drain and Boyd (1935a, 1935b) examined over 100 children without finding any correlation between the incidence of caries and concentrations of calcium and phosphorus in blood and saliva. They suggest however the possibility of a relationship between the retention of calcium-phosphorus and immunity to caries. Becks and Wainwright (1934, 1938) and Becks and Simmonds (1935) reviewed and criticized extensively the technical procedures described by other investigators for the determinations of calcium and phosphorus in saliva. They report certain slight modifications in the methods of Halderson and Bergen and Kirk and Schmidt for calcium estimations, as well as a modified Bodansky method for phosphorus.

Quantitative determinations of salivary calcium, phosphorus, sugar, hydrogen ion concentration (potential acidity), and the total neutralizing power (the sum of actual acidity and alkalinity) have been reported by several laboratories. Probably one of the chief reasons for the glaring disagreement in results is the difficulty of securing representative samples of saliva. It has been emphasized many times that the innervation of the salivary glands is so complicated that diverse unpredictable stimuli may produce profound alteration in type and rate of

secretion. Until standard methods of collecting salivary samples can be devised and adhered to by all laboratories, the progress in this direction will be slow and barren of results.

The preceding sections dealing with the anatomical, bacteriological, and chemical factors in etiology have described in part the various effects observed by experimenters in their respective fields. The following section is restricted to a discussion of a few typical experiments selected to focus attention upon the effects arising from differences in mineral and vitamin intake as reflected in alterations in the osseous system, in dental development, in the incidence and in the treatment of caries. Researches on the effects of diet in relation to pathogenesis and prophylaxis of caries comprise first, laboratory studies in which various groups of animals were maintained on diets designed to favor either the development of caries-like lesions, or dental hypoplasia, or alterations in oral flora, particularly the elaboration of aciduric organisms; second, clinical studies on groups of children dealing with prophylaxis rather than pathogenesis. The majority of the experiments present perplexing questions for which the answers are too involved for clarity. In spite of the apparent disagreement one may follow a thread of concurrence through this dietary maze, even though it be composed unfortunately in some cases, of experiments futile in concept and lacking scientific accuracy.

The initial effect of a diet deficient in vitamin A, demonstrable in the incisal teeth of rats and guinea pigs, is atrophy of the ameloblasts and subsequently of the entire enamel organ. In rats, metaplasia and decalcification supervene, but in guinea pigs ossification results (Smith and Lantz, 1932; Wolbach and Howe, 1933). These changes in the developing enamel have their counterpart in dentine hypoplasia and in pulp metaplasia (Marshall, 1923, 1927a, 1927b, 1928, 1931, 1935).

More remarkable, however, are the observations of Orten, Burn and Smith (1937). Rats surviving about 365 days' restriction in amount of vitamin A develop odontomata in the region of the incisor teeth but not in the molar area. In some instances supernumerary incisors were formed and frequently the original incisors became distorted as a result of the tumor growth. Arnim and co-workers (1937a, 1937b, 1933, 1936) by restricting the mineral intake of rats produced resorption and rarefaction of the alveolar process, an increase of the fat of the bone marrow, irregularities in dentification and occasional caries. When the diet was restored to normal levels these changes were reversed to the

extent that osseous structure was rebuilt and secondary tubular dentine formed.

McCollum (1931) pointed out that a diet lacking each vitamin will produce deleterious effects upon dental tissues and that defective diets are responsible for hypoplasia or metaplasia after eruption of the tooth. Thus defective diets alter dental structures from within by changing the character of the dentine. Although a low Cevitamic acid intake produces startling changes in odontoblastic function, the emphasis which has been placed on this vitamin is not justifiable.

Rat caries said to be identical with human caries has been described by Blackberg and Berke (1932). Animals receiving a basal ration plus a therapeutic amount of vitamin D for 150 days were free from caries. Without vitamin D, one-third developed caries and when an excess of viosterol was administered, all animals had carious teeth. Histopathologic changes beginning with a low grade hyperemia progressed through the various degenerative stages to pulp necrosis. It was suggested further by the authors that circulatory disturbances in the pulp may lead to decalcification of dentine (internal resorption).

Lilly (1932, 1938; Lilly and Grace, 1932; Lilly and Wiley, 1934) stated that white rats on a rachitogenic diet developed florid rickets with extreme bone and joint deformities. No dental caries was observed. The same laboratory found no caries on high carbohydrate or high fat intake, but one hundred per cent caries in rats 125 days old fed on diets containing coarsely ground yellow cornmeal. The same diet became non-carious if the corn was cooked or if the coarse particles were further ground to pass a 60-mesh sieve. Rosebury and co-workers (1932, 1933, 1934) produced fissure caries in rats, histologically similar to fissure caries in man, but this lesion did not develop in animals younger than 100 days. Addition of calcium, phosphorus and cod liver oil to the deficient diet was without effect. They reported also that they were unable to demonstrate any relation between incidence of caries and the relative amounts of calcium and phosphorus in the blood and in the ash of teeth and bones. Similar observations were made by Bibby and Sedwick (1933). They compared the results produced on teeth by feeding three types of experimental diets: first, a diet of yellow corn, wheat gluten, calcium carbonate and sodium chloride gave the highest incidence of caries; second, a heterogeneous diet of hospital scraps resulted in relative immunity; third, the addition of cod liver oil to a deficient diet was without noticeable effect.

Shelling and Asher (1933) likewise report that the incidence of caries

is high in rats maintained on coarsely ground corn. It is infrequent on diets poor in this grain or in those composed of casein and starch, or of grains having particles finer and softer than corn. Although no relation could be determined between caries and sex in animals, the authors confirmed a fact mentioned earlier by Marshall (1927a) that the lesion is infrequent in young animals, especially those that have been maintained on a deficient diet for less than two months. Furthermore it is probable that there are etiological factors in rat caries which are inapplicable to man.

The clinical application of the experimental dietary studies has met in some instances with gratifying success; other reports have described only failure. Earnshaw (1936) presented statistical data in support of the thesis that caries is an index of malnutrition. A different viewpoint has been suggested by Osborne (1933) who has observed that the incidence of dental caries is as high in Australia as in other parts of the world, in spite of the fact that the diet in that locality is rich in vitamins. That the problem cannot be limited to a study of vitamins is indicated also by Day and Sedwick (1934a) who have stated that the administration of vitamin concentrates is without effect in reducing the incidence of caries.

As is pointed out by Ker (1936) it is doubtful that the term "odontoclasia" of Jones (1935; Jones and Crosland, 1936) is justified, for the reason that no proof has been advanced to show that this so-called condition is other than hypoplasia or rampant caries. It is said to be the result of a low alkali intake. The lack of convincing evidence in this study is unfortunate, because one is prone to accept with enthusiasm any advance in theories of etiology, pathogenesis or treatment, however vague the newer ideas may seem.

Some of the theories of Mellanby have been received coldly but others deserve more consideration. After recounting the experimental studies the conclusion is drawn, which has often been mentioned before, that dental hypoplasia results from deficient diets. One speculation, which is difficult to follow, is that the nature of the food during the developmental period determines whether the teeth later will show caries or pyorrhea. It is said that of the usual foodstuffs, oatmeal exhibits the greatest "decalcifying" power and white flour the least. An anti-calcifying factor is postulated ("Toxamin"), which is assumed to interfere with the normal development of teeth. Although oatmeal and wheat germ contain more calcium and phosphorus than some of the other cereals, the fact that these elements are present as the relatively

insoluble inositolphosphate makes them less available (Bruce and Callow, 1932, 1934). A special committee of the Medical Research Council, of which Mrs. Mellanby was a member, in reviewing the experimental data presented by her from 1928 to 1936, has reported (1936) first, no definite evidence has been developed which indicates that diet exerts any effect upon the structure of permanent teeth; second, a high vitamin D intake during the years of dental formation and growth and before the full eruption of the permanent teeth, diminishes the incidence of caries, especially in premolars and second molars, but is of less effect on first molars (and presumably on incisors). Thus again experimental evidence has been advanced supporting the theory of the close parallelism of hypoplasia with caries (Marshall, 1938).

Kugelmass et al. (1934, 1936) state that children free from dental caries consistently show a dietary intake containing a preponderance of raw fruit and vegetables. This seems to be an important observation and a step in the right direction. It is quite possible that a study of caries-immune rather than of caries-susceptible individuals may disclose nutritional and endocrine balances exerting a greater regulatory effect than has been suspected.

Hess and Abramson (1931) question seriously the assumption made by some investigators that dental caries is a specific nutritional disorder. Similarly, Rosebury and Foley (1934) suggest that caries is not necessarily due to defective tooth structure. A survey of 319 children conducted by Brodsky (1933) failed to show any relationship between the level of calcium or phosphorus and the development or arrest of caries. Two of the statements are equally confusing, first, that "children with marked hypoplasia exhibited a low caries incidence," and second, that "ingestion of candy and sugar seemed not to be an appreciable factor." These observations are not in accord with those of other research workers in this field. McBeath (1932) compared dietary effects upon three different groups of children from three different orphanages and reached the following conclusions: first, no one dietary factor exerts a predominating influence over any other factor; second, control of caries by a directed regimen is possible.

The studies of Hanke (1933) have been reviewed extensively in the Journal of the American Medical Association (see: Hanke, review, 1934). It was pointed out in detail that conflicting statements in addition to inadequate data, both clinical and experimental, make this work seem a gesture rather than an accomplishment. His experiments, unfortunately, must be dismissed with the same comments as those which

apply to Davis (1930), namely, that the clinical and laboratory data are insufficient and inadequate.

Clarke and Smith (1935) confirmed an observation which has been reported many times: that a limited mineral intake is reflected by retarded somatic growth. When the normal diet is substituted for the restricted one, growth is resumed.

Day (1934) and Day and Sedwick (1934b) conducted clinical studies on the influence which fat soluble vitamin A may exercise on the development of dental caries. To the ordinary diet, without attempting to regulate food intake, there were added six thousand U.S.P. units of vitamin A and fourteen hundred Steenbock units of vitamin D. Comparing one hundred and seventy-one controls with one hundred and forty-seven experimentals, the authors were of the opinion that the experimental group showed no reduction in caries incidence, as judged by average increase in percentage of infected teeth. An experimental period of fifteen months is probably inadequate for an age group of thirteen years. It will be recalled that the British Anthropometric Society presented figures to indicate that the greatest growth rate of the human occurs between birth and four years of age. This is also the time of greatest rate of dental development. The conclusions, therefore, of Day and Sedwick were really to be expected when one considers that the greater amount of dental development had already been accomplished at the beginning of the experiment.

Eddy (1934) discussing the Boyd, Drain, Nelson diet says it "improves tooth resistance to decay in test groups when contrasted with controls on regular orphanage regime." This diet increases the phosphorus intake from four to ten per cent, and all known vitamins, particularly A and D. Boyd, Drain and Stearns (1937) have greatly extended their earlier studies and have lately (1937) described a clinical experiment in which five subjects between three and six years of age were maintained on various dietary modifications for a total period of seven months. During this time frequent clinical examinations determined the extent and rate of decay, and caries was considered active when the exploring tine located an area of exposed dentin. The authors reported that a diet adequate in all respects except vitamin D reduced, but did not stop, the development of caries. When 600 units of vitamin D per day were added for nine weeks, the rate of caries development was arrested. It was emphasized that a considerable period must elapse after starting vitamin D therapy before caries control can be accomplished. Of more importance than this, however, is the state-

ment that almost complete arrest of caries may be obtained by dietary supervision.

Realizing the fallacy of drawing comparisons too closely between animal experimentation and clinical experience, one may yet be justified in pointing out that the present situation in the epidemiology of human caries may be the indirect result of deficient diets and particularly those classified by McCollum as "borderline." Some support is given this premise when it is recalled that laboratory experiments produced lesions resembling caries, when animals were maintained on deficient diets for relatively long experimental periods (with rats, six to eight months). However, with second and third generation animals so controlled, this result is more easily gained (Marshall, 1933). Perhaps it is not too far-fetched to suggest that the human race today is suffering from dietary insufficiencies of former generations. Caries is only one of the least of these unfortunate results.

In the field of endocrinology speculation has far outdistanced accomplishment. While the importance of normal glandular function for calcium utilization is readily recognized, it must be pointed out again that the influence of the ductless glands is probably less direct than other factors concerned in the etiology. Glandular dysfunction may be reflected in terms of dental hypoplasia, agenesis, malformation or malposition. Attempts to correct dystrophies in either osseous or dental systems by the administration of various biologic products have not been sufficiently studied to permit definite conclusions. Only a few scattered cases have been reported in which any known improvement in bone formation has been observed. Furthermore, there is less corroborating evidence dealing with alveolar bone than with other parts of the osseous structure. Thus it is evident that after eruption of teeth, endocrine dysfunction may contribute to dental malposition but not to dental malformation.

Jung and Skillen (1929) working with thyro-parathyroidectomized rats, described changes in enamel epithelium of an atrophic nature, confirming and extending Erdheim's experiments but contradicting Gies' comments (Gies and collaborators, 1930). Schour and Van Dyke (1932a, 1932b, 1933) have reported that hypophysectomized rats showed a progressively slower and slower rate of eruption of incisors. Eventually eruption ceased. Upon administration of pituitary growth-promoting hormone, there was a slight increase in the rate of eruption. When given to normal animals, however, no effect on eruption rate was

found; only an increase in the body weight of the animal could be recorded. Gordon and Kuskin (1935) have studied the relation of glandular dysfunction to irregularities in time of dental eruption. In the normal child the beginning of teething, walking, and talking appears related to the thyroid and the anterior pituitary growth hormone. Delay in appearance of these attributes may serve as an indication of glandular abnormality, provided that other non-endocrine factors have been excluded. Reduction in blood calcium and a slight increase in the inorganic phosphate result when secretion of the parathyroid hormone is interfered with by para-thyroidectomy. In cases of hyperparathyroidism the bone changes are those of cystic fibrosis, but variations in tooth structure have not been reported.

The effects produced on calcium metabolism by glandular changes have been studied mainly from alterations in the skeletal system, but the dental aspect either has been ignored or else treated too superficially to be of value etiologically, pathologically, or therapeutically. Barrows' studies (1938) upon parathyroid extracts have shown that when these preparations are administered, the hyperparathyroidism so produced is characterized by an increase in blood acidity. As a result alkaline salts are dissolved out of the bone and osteoclasts phagocytose the salts (osteitis fibrosa). Thus osteoclastic activity is secondary to bone resorption. Continued injection eventually gives rise to an opposite reaction and "marble bone" is formed. The degenerative changes in the osseous system probably find a counterpart in hypoplastic dentogenesis.

Experiments of this kind stress the importance of adequate calcium utilization. All efforts directed toward an optimum diet are fruitless unless the various factors or circumstances contributing to transport and normal deposition of the several inorganic salts can be recognized. Unfortunately, the physiology as well as the pathological physiology of the endocrines is not fully understood and until further facts can be discovered, progress in this phase of etiology will be uncertain.

It was the opinion of the White House Conference on Child Welfare (1930) that no method of treatment has yet been devised which will either prevent or repair the ravages of dental caries. In spite of this pessimism, sufficient evidence is accumulating to warrant a distinctly optimistic attitude in regard to future methods of treatment.

It is well known that filling operations are not entirely satisfactory. A careful examination of 8,500 extracted teeth showed that 5,435 or

about 60 per cent were carious (Marshall, 1937). Of these there were 1,746 or about 32 per cent which showed secondary caries under or around the margins of fillings. It is this latter figure which is more significant than first appears. The factors contributing to the incidence of secondary caries comprise all those previously mentioned in the etiology and in addition include inherent difficulties in operating, in sterilizing the cavities preparatory to filling and the undesirable physical and chemical properties of the filling materials. McLean (1935) is accurate when he says that fillings of a given classification are not uniform in chemical and physical qualities and therefore do not always prove satisfactory from a standpoint of caries prevention.

Brekhus and Armstrong (1936) suggest that cod liver oil, orange juice and tooth brushes alike have failed to prevent caries. Furthermore, "When human beings were using their teeth and were dependent upon them for existence, nature furnished them unstintingly. There was no caries and no pyorrhea." It is hardly necessary to point out in this connection that anthropologists and Egyptologists overwhelmingly refute this inference. The statistics of caries presented by Brekhus and Armstrong show only one part of the whole problem. To make their data of value it would be necessary to examine the same individuals after five years, ten years, and fifteen years. The figures would then present a true perspective which now is lacking. Suk (1936) indicts the plan of oral hygiene by saying that the races with the worst teeth do the most tooth brushing and mouth washing. He could have done better if he had added that these same races are nearer to dietary insufficiencies than other races.

Kugelmass (1936) suggests a ten point plan for the clinical control of caries in which are included not only the recognized regimen of public health control, immunization, and physical measurements, but also dietary supervision and periodic dental examination. Stone (1936) invites the physician to coöperate more closely with the dentist in an effort to control early ravages of caries in deciduous teeth.

Klein (1938a, 1938b) classifies for convenience teeth as decayed, missing, filled, and presents tabular data in support of the thesis that dental caries in childhood constitutes a health problem of major importance. The plan for providing more adequate dental care is based on a proposal that all dental defects in permanent teeth be treated in children of five to six years of age who attend the first grade in school, and also, each succeeding group of first grade children. As these students mature, the previous operations may be checked or repeated and

any new cavities filled. The plan requires the full coöperation of the community, the school authorities and the dental profession alike.

The rôle of dentifrices, tooth pastes, mouth washes, deodorants and in general all preparations or solutions either of true or alleged antiseptic value is insignificant in therapy of caries. None is effective, for rapid dilution by saliva renders them valueless except for lavage or momentary stimulus to glandular action. But a new study on caries prevention is being made in several localities. Ammoniacal silver nitrate is applied on small areas not yet carious, but so situated on tooth surfaces that they lie in the susceptible zones, not easily accessible for the tooth brush. This plan has many advantages but the results of the treatment cannot be assessed for several years (Prime, 1936). Some preparations, mentioned as antiseptic agents for renal infections, have also been suggested as suitable for oral therapy. Although other laboratories have had less success than Bunting, Hadley, and Hard (1930, 1936; also Bunting, Delves and Hard, 1930), these workers regard hexylresorcinol as the most effective bactericide in the prophylactic treatment of caries. The supplementary dietary measures provide for adequate proportions of milk and green vegetables, and the reduction of sugar consumption to a physiological minimum.

After an exhaustive review of the literature (1936) the Council of Dental Therapeutics was forced to conclude that there is no evidence available to warrant the use of calcium-phosphorus compounds for the prevention and treatment of caries. The claims of the manufacturers and of the various essayists are unfounded and such products lack a proven value as therapeutic agents. East (1938) reports satisfactory results based on follow-up data on dietary studies conducted on children in institutions. By following the suggestions of Boyd and Drain, caries incidence was lowered. Privitera (1938) also confirms these results.

Caries therapy has advanced during the last few years, first, by focussing public attention on the seriousness of the problem; second, by devising a quick and easy method of prophylaxis of susceptible areas; third, by instituting dietary supervision whenever possible; fourth, by improving the physical properties of the filling materials. The high incidence of this unfortunate disease is not generally understood, nor its sequelae. Any plan for intensive coöperative research should include studies in all of the various factors which have been discussed, bearing in mind that agencies which favor normal dental development demand intensive investigation.

SUMMARY

Regretfully one is forced to acknowledge that most of the factors which have been tested have been confined to selected groups of individuals. Suppose a comparison of rachitic and non-rachitic groups indicated a correlation of caries and hypoplasia in the first set of persons but not in the second. What about the other etiological factors? In some of the experiments it appears that the errors produced by "random sampling" have not been considered nor the different hereditary influences assessed.

It is evident that the contributions within the last fourteen years to the etiology, pathogenesis, prognosis and therapy of caries are of immense value from the standpoint of prevention. Anatomical defects in teeth are of histologic as well as pathologic importance. Therefore the secondary factors—dietary, endocrine, hereditary—contributing to the formation of poorly calcified teeth assume greater importance than in the past. Progress also in the solution of other problems relating to local conditions (bacterial, chemical) is of such importance (the pyruvic acid hypothesis, for instance) that the theories of Miller again must be revised to include the suggestions of Hansen, Fosdick et al. upon the local elaboration of acids other than lactic acid by definite bacterial symbiotic relationships hitherto unsuspected.

Caries may be treated now more successfully than ever before; not only has dietary supervision reduced the number of carious teeth but even through the periods of pregnancy and lactation the lesions may be controlled to some extent. Both mother and child respond to the regimen. In addition the wider application of ammoniacal silver nitrate solutions, although delayed twenty years, promises much in the control of the earlier stages of dental decay.

In spite of the advance in the therapeutics of caries, the etiology remains an enigma. No explanation of immunity to caries has been advanced; the lack of decay among the inhabitants of the island Tristan Da Cunha in spite of the high carbohydrate content of the diet, is certainly an incontrovertible argument against the theories now accepted. Rampant caries occurs and disappears without aid or hindrance of physician or dentist. These are only a few of the puzzles awaiting solution. Satisfactory answers must be found for the following queries:

1. Why does caries show a definite age incidence, for example, at adolescence? One suggestion is that the atrophy of the thymus gland at puberty, coincidental with the activity of the sex glands, may tem-

porarily upset the calcium-phosphorus balance and presumably also affect adversely the phenomena of dentogenesis.

2. What relation exists between the amount, content and acidity of salivary secretion, and caries? No reports on hydrogen ion concentration are satisfactory and too much confusion exists in relation to buffer value.

3. What are the relationships between blood calcium and blood phosphorus and the corresponding salivary percentages? The answer "dietary deficiencies and endocrine unbalance," is too vague.

4. Is there any relation between salivary mucin and rapid plaque formation? None has been proved.

5. How may malnutrition, both dietary and endocrine, be studied from a more comprehensive dental viewpoint?

6. What new methods of treatment, such as filling, sterilization of initial lesions, or diet regime may be devised for further control of this disease?

Caries affects 90 per cent of the population of the civilized world; it requires research in the exact and experimental sciences, and its conquest is a challenge to the physicist, chemist, dentist, anatomist, bacteriologist, physiologist, physician, pathologist and biochemist.

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THE MINERAL METABOLISM OF NORMAL INFANTS

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The inorganic elements of the body may be classified according to their manner of use. Calcium is deposited chiefly in the skeleton, phosphorus and magnesium occur both in skeleton and in the soft tissues. Chloride, sodium and potassium are associated chiefly with the amount of body water and its distribution between intra- and extracellular fluids. Other minerals, as iron and iodine, vary in amount with the metabolism of the special tissues with which they are concerned.

Knowledge of the metabolism of inorganic elements in infancy requires primarily a study of changes in inorganic composition of the body during a period of very rapid growth, and is concerned equally with changes in composition occurring both before and after birth. The chemical composition of the fetus at different stages of gestation has been studied by direct analysis. Changes in composition of the growing infant must be studied by indirect methods, the customary procedure being that of Lindberg, who estimated from metabolic studies the quantity of substance retained during the time required for the infant to gain 100 grams in weight. This method of study is open to several obvious errorrs, but nevertheless is of considerable value, particularly in comparing the effects of dissimilar dietary regimens on body composition.

Moulton studied changes in body composition of mammals, including humans, during growth and observed that, if calculated on a fat-free basis, changes in nitrogen, ash and water conformed to definite laws, the water content varying inversely with the nitrogen and ash. Yannet and Darrow have brought forward evidence that the changes in composition of separate tissues during growth conform to the law of constant differential growth ratio as defined by Huxley; Hamilton has applied the same law to the changes in total water content of the growing human fetus. If the law holds for body water, it presumably holds also for the changes in body composition in regard to other substances, particularly those like calcium, which are added to the body by simple accretion and utilized almost entirely by one type of tissue.

Calcium. As over 98 per cent of body calcium is found in bone, a discussion of calcium metabolism is a discussion of skeletal growth and mineralization. Analyses of the calcium content of fetuses of various ages are numerous and show that the relative, as well as the absolute body content of calcium increases steadily throughout the fetal period (chart 1) so that at birth, the body of the normal infant of 3000 grams weight contains about 25 grams of calcium. If the mother's body is severely depleted of calcium during pregnancy, as in osteomalacia, the body of the infant may contain much less than the normal amount of

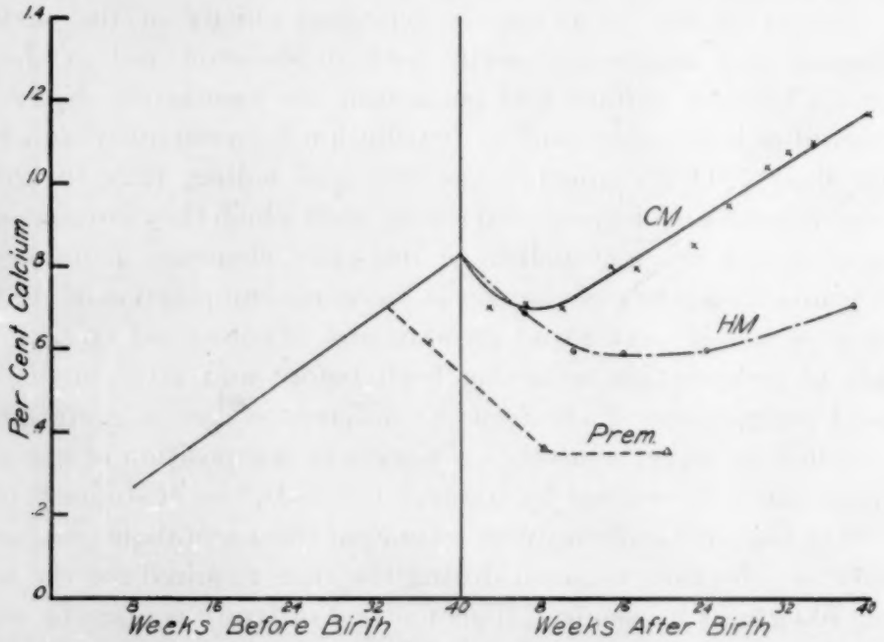

Chart 1. Changes in relative calcium content of the fetus and infant. The regression line of calcium content of fetus is drawn from data in the literature. *CM*—infants fed cow's milk; *HM*—infants fed human milk; *Prem.*—prematurely born infants fed human milk.

calcium and fetal rickets may occur. The evidence as to the effect of less severe calcium undernutrition of the mother on the fetal content of calcium is more conflicting. Booher and Hansmann could find no difference in the percentage of calcium in fetal bone when the mothers' diets varied widely in calcium content. On the other hand, Toverud has reported increasing incidence of soft skull and early rickets in infants born of mothers whose diets had been low in calcium, and Sontag reports roentgen-ray evidence of poorer bone structure in such infants. Increasing numbers of reports of rickets in very young infants are found in the literature. According to Toverud, the incidence of prematurity also

increases with poor maternal nutrition. Diets high in calcium, phosphorus and vitamin D during pregnancy are known to result in increased serum calcium and inorganic phosphorus levels of the fetus and are said to result in fetuses with greater than average density of bone. The possibility of an effect of diet in pregnancy on bone size of the infant cannot be disregarded. Undoubtedly the fetus grows at the expense of the maternal tissues, but evidence seems to be accumulating that this mechanism acts only within limits, and the maternal diet, especially in its calcium and vitamin D content, exerts a real effect on the skeletal development of the fetus.

Assuming that the calcium content of the body of the infant is within normal limits at birth, the amount of calcium retained daily by the infant will depend largely on the daily calcium intake and the quantity of vitamin D ingested or received through ultra-violet irradiation. Because of space limitations, discussion will be confined chiefly to the extremes of intake permitted by customary dietary regimens—human milk without added vitamin D and undiluted cow's milk with an added source of vitamin D.

The composition of human milk varies widely among individual women and is dependent, at least in some measure, on the adequacy of the diet. The reported values for calcium content range from 68 to 717 mgm. to the liter, with an average value of about 300 mgm. to the liter. The young baby ingesting about 650 cc. of milk receives only about 200 mgm. of calcium daily. Very little calcium is excreted in the urine of such an infant; the minimal fecal excretion, regardless of intake, seems to approximate 150 mgm. Retention of calcium during this early period must be low; the reported values average 48 mgm. daily. As the infant grows and ingests more milk, the amount of calcium ingested and retained increases until the infant of eight months ingests about 350 mgm. and may retain nearly 200 mgm. of calcium a day. The average rate of weight gain of a breast-fed infant is 25 grams daily to 3 months of age, 20 grams daily from 3 to 6 months, and decreases to 15 to 17 grams a day during the last half of the first year. Using these weight gains and data reported in the literature for daily calcium retention of infants fed human milk, the changes in body content of calcium as estimated by Lindberg's method would be as shown in chart 1, curve HM. The percentage of calcium in the body decreases very sharply during the rapid growth of early infancy and then remains constant at a value approximately equal to that of the 6th to 7th fetal month until the infant is 6 months of age or older. During later infancy

with its lower rate of weight increase and larger daily intake of calcium, the percentage calcium content of the body increases slowly, but not until the last quarter of the year is the birth value regained. In prematurely born infants fed human milk only, as is seen from the chart (curve Prem), the reduction in per cent of body calcium is still more drastic, because of the smaller amount present at birth and the low daily intake of calcium. The rate of growth of this group was estimated as 25 grams daily—more rapid growth would result in still greater rate of decrease in relative body calcium content. It is not surprising that prematurely born infants who have been fed only human milk almost invariably develop rickets.

Few data are reported concerning the calcium retention of infants fed human milk and given vitamin D. Such infants seem to show less variability of retention and greater average retention of calcium than infants not given vitamin D. The possible amount of retention, however, is limited by the low intake.

Infants fed diluted cow's milk feedings without added vitamin D show extremely variable, and usually poor retention of calcium. The prevalence of rickets in these infants is evidence that the relative calcium content of the body probably decreases even more rapidly than that of infants fed human milk.

At the other end of the feeding scale is the modern practice of giving undiluted cow's milk treated to give a fine curd in the infant's stomach, and a source of vitamin D. This dietary regimen provides about 3 times as much calcium as does human milk and has the added provision of vitamin D for efficient utilization. The average daily retention of calcium by infants fed cow's milk and 350 to 400 U.S.P. units of vitamin D daily, varies from 100 mgm. for infants under one month to 300 to 400 mgm. for infants from 4 to 10 months of age. During a large part of infancy, the daily calcium retention of these infants is thus as great or greater than the daily intake of the breast-fed infant. The effect of this increased retention on body composition of calcium is shown in chart 1 as the curve *CM*. The rate of weight gain was determined from the actual weights of the infants of the reported studies. It will be observed that even with the marked increase in calcium intake, the infant under 3 months of age still shows a rate of increase in the body content of calcium less than the increase in weight. After 3 months of age the rate of increase in percentage of body calcium approximately parallels that during fetal life and produces a curve similar to that predicted by Moulton for rate of increase in ash content of the fat-free body.

Studies of relation between calcium intake and retention indicate that a direct relation exists between the two at all levels of milk intake studied, and offer no evidence that the maximum possible retention has been attained at any age during infancy. Studies of infants fed calcium salts in addition to milk, or calcium salts as the sole source of this element show that calcium from inorganic sources is well utilized, especially when the food is alkaline in ash and a proper balance between calcium and phosphorus intake is maintained. Short-term studies are reported wherein the total calcium intake is much greater than the amount supplied by cow's milk. These studies indicate also that retention tends to parallel intake. No studies have been reported wherein infants have been given these larger quantities of calcium over sufficient period of time to have attained a stable daily retention. The data are sufficient to show, however, that with the aid of vitamin D, the capacity of the infant to absorb and retain calcium is far greater than that permitted by the amount of calcium provided in human milk.

Other factors than calcium and vitamin D intake affect the amount of retention of calcium. Illness, whether febrile or not, decreases the calcium retention of both breast and artificially fed infants. An increased intake of roughage decreases the retention to some degree, though it seems probable that such decreases are usually transitory. Acid-ash diets are not usually a factor in infancy; however, the ingestion of fruits or vegetables containing oxalates or other organic acid ions whose calcium salts are insoluble will decrease the calcium retention of the infant measurably. The addition of lactose to the diet is reported to increase the calcium retention of young animals ingesting a diet low in calcium. Each infant given milk is ingesting some lactose; whether an additional amount would cause a further increase in the retention of calcium by the infant, remains to be determined.

The mode of excretion of calcium is affected by the diet. Babies fed cow's milk or calcium salts of equal alkalinity generally excrete larger amounts of calcium in the urine than do infants given only human milk. The amount excreted for a given intake varies considerably in individual infants and seems to bear little relation to the amount retained. The maximum daily urine calcium reported for an infant is 286 mgm. daily, excreted by an infant fed a large excess of calcium carbonate; usually the 24 hour excretion is under 20 mgm. Calcium excretion through the skin is negligible.

The serum calcium of the infant at birth is of higher concentration than that of the mother. It may vary from 10 to 13 mgm. per 100 cc.,

but if the mother's diet has been adequate in calcium and vitamin D, is usually above 11 mgm. per 100 cc. For both breast-fed and artificially-fed infants given sufficient vitamin D the serum calcium tends to remain constant throughout the period of infancy at a value of about 1 mgm. below the average level for newly born infants. The normal range for infancy is usually given as from 10 to 12 mgm. per 100 cc.; infants receiving ample vitamin D tend to have serum calcium levels nearer the upper limit of normal, but when the vitamin D intake is barely adequate, the serum calcium approaches 10 mgm. per 100 cc.

The marked difference between the calcium retention of infants given high calcium feedings plus vitamin D and those fed human milk has aroused much discussion as to the question of "supermineralization" of the infant fed cow's milk. Attempts to study the question by sudden dietary changes from the low to the high calcium feeding, and reverse, lead to unsound conclusions, because of the relatively long period of time required to stabilize an infant to a different type of feeding. Rominger's experiment wherein the calcium retention of an infant was studied for 56 consecutive days without evidence of any loss from the body demonstrated conclusively that the infant is able to maintain a consistently high retention of calcium for a long period without intervening periods of loss. The fact that, even with the high retention, the young infant does not quite maintain the proportion of calcium present in the body at birth may be construed as evidence against any excessive amount of deposition.

The curves for percentage of body calcium in infants given human and cow's milk are so dissimilar that either one must assume marked differences in relative proportion of skeleton to body weight (or in amount of fat deposits) or a very wide range of "normal" calcification of bone. The changes in percentage of calcium would necessitate an average decrease in the breast-fed infant of at least 5 per cent in the relative proportion of body weight due to skeleton if the calcium content were even maintained constant. Anatomical data, however, show that the wet weight of the skeleton remains practically a constant proportion of body weight during infancy. For the differences to be attributable to differences in fat content, nearly 70 per cent of the weight gain of the breast-fed infant would be due to fat. A true decrease in calcium content of bone must be assumed. Eliot and Park state "the long bones of the extremities of the healthy fetus at term are almost completely filled with cancellous bone. The marrow cavity does not become well developed until some time after birth. The meaning of this must be that under normal conditions the fetus is born with a store of calcium in the

skeleton." Hamilton has construed this observation to mean that the relative depletion of body calcium in early infancy is normal and is a utilization of excess stores. Against this conclusion is the occasional occurrence of clinical rickets of moderate or greater severity among breast-fed infants and the common occurrence of physiological osteoporosis. The maintenance of a calcium reserve also may prevent severe depletion of the skeleton during illness.

The well-known fact that rickets develops in the growing infant demonstrates the fact that skeletal growth is not wholly dependent on repletion of calcium stores. A comparison of the rates of linear growth of infants whose skeletons contain larger amounts of mineral shows that, while depletion of body stores may not stop growth, the rate of growth increases with the degree of repletion. The rate of growth of infants given 350 to 800 U.S.P. units of vitamin D daily is greater than that of infants fed not over 135 units daily; the babies of the latter group grow faster than infants given no added vitamin D. The daily calcium retention of the 3 groups is in the order of their rates of growth. Both the rate of linear growth and the retention of calcium decrease when the infant is given 2000 units a day or more of vitamin D.

Notwithstanding the increased skeletal content of calcium in infants fed cow's milk and 350 to 800 U.S.P. units of vitamin D, it has been reported that such infants often show deviations from the normal bone pattern at the epiphysial junction. These deviations appear to be similar to those observed in infants not given vitamin D except that in the latter group, such irregularities seem to precede clinical rickets, whereas in the group retaining more calcium, the irregularities are transitory, appearing during the period of most rapid growth and disappearing without change in dietary regimen. This fact has led to much confusion in the literature concerning rickets. Much further study is needed correlating mineral retention and rate of skeletal growth with studies of relative size and density of bone, as well as bone patterns as shown by roentgen ray.

Phosphorus. Body phosphorus is distributed between the skeleton and the nitrogenous tissues. The amount in the skeleton varies with the skeletal content of calcium, the ratio of calcium to phosphorus being reported as 2.21 to 2.23:1. The ratio of nitrogen to phosphorus varies with the different tissues, being about 15.3:1 for muscle and 13:1 for liver, due to the increased lipoid and nucleoprotein content of the parenchymatous tissues. A ratio of 17:1 is often used for the nitrogenous tissues of the body as a whole.

The relative changes in body phosphorus, nitrogen and calcium are

thus interdependent. The percentage changes in calcium have been discussed. The percentage of nitrogen in the fetus increases as steadily as that of calcium throughout gestation (chart 2), therefore the relative phosphorus content must increase likewise. From analyses of full-term fetuses, at birth the body of a 3000 gram infant contains about 60 grams of nitrogen, 25 grams of calcium and 14.5 grams of phosphorus. If the ratio of calcium to phosphorus in bone is 2.23, 11.2 grams of phosphorus would be contained in the skeleton and only 3.2 grams in the soft tissues, making the ratio of nitrogen to phosphorus 18.8 for the

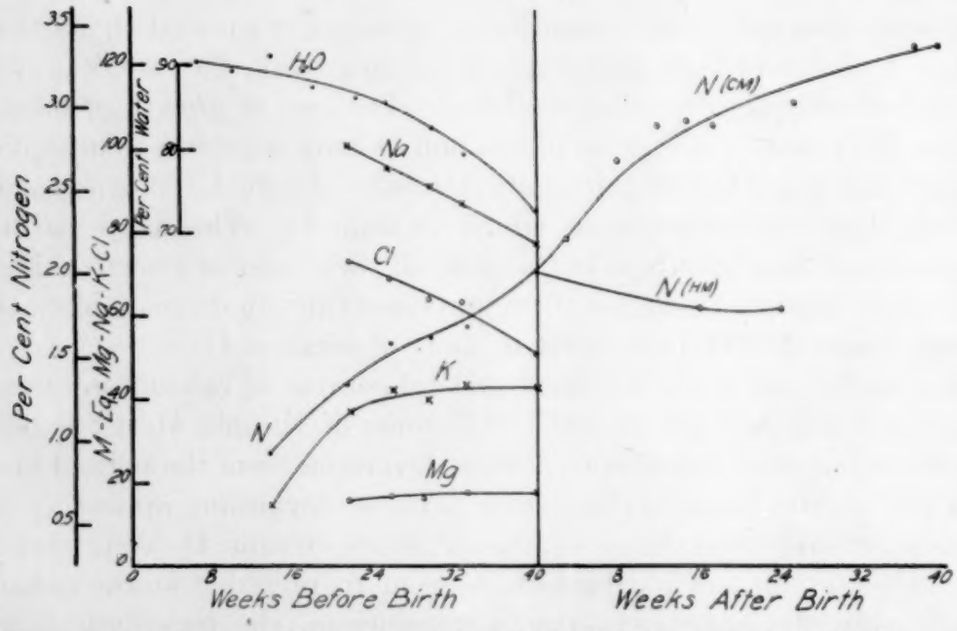

Chart 2. Changes in nitrogen, water and electrolyte content of the fetus, and in nitrogen content of the infant. *CM* and *HM*—infants fed cow's and human milk respectively.

newborn human infant. The fetus at 24 weeks contains relatively more phosphorus in proportion to the nitrogen, the ratio of N:P of soft tissue being 16:1.

The rate of increase in nitrogenous tissues after birth will depend largely on the protein intake. Here again the extremes in customary feeding practices are the low protein feeding of human milk and the high protein intake permitted by the feeding of undiluted cow's milk. The per cent of nitrogen in the body of a breast-fed infant remains at or decreases slightly from the birth value of 2.01 per cent; that of the infant given cow's milk tends to parallel the curve predicted by Moulton for the fat-free body. Changes in both nitrogen and calcium composition

of the body are thus very similar for the same dietary regimens; changes in phosphorus content will follow changes in the other two elements.

The low phosphorus intake of the infant fed human milk results in the retention of a large percentage of the material absorbed. Little if any phosphorus is excreted in the urine. On the other hand, the amount of phosphorus in cow's milk is not only much greater in absolute amount but is greater also in proportion to the calcium and nitrogen content. The proportion of amount absorbed to amount retainable is greater than for human milk. As a result, the kidney becomes the chief organ for excretion of phosphorus, 60 to 70 per cent of the total excretion being in the urine. No evidence of damage to the kidney by this increased phosphorus excretion has been presented.

With phosphorus and nitrogen, as with calcium, the quantity retained by the infant is directly proportional to the intake, if the food is in a form well utilized by the infant. No evidence of maximal absorption or retention has been reported. Thus, the amounts of protein, calcium and phosphorus which can be ingested by an infant fed human milk are far lower than the quantities which that infant is able to digest, absorb and retain. Discussion of compensatory factors is outside the scope of this article.

Blood phosphorus values differ materially in infancy from the values observed in later childhood. At birth, the serum inorganic phosphorus, like the serum calcium, is greater than that of the maternal serum; average values reported vary from 3.5 to 5.4 mgm. per 100 cc., the larger values being observed in blood from infants whose mothers had ingested good diets during pregnancy. The serum phosphorus increases during early infancy, and if the intake of vitamin D is ample, by 6 months of age the maximal values are attained, from 5 to 7 mgm. per 100 cc. The range of serum values is the same for breast-fed and artificially fed infants. By one year of age, serum inorganic phosphorus varies between 5 and 6 mgm. per 100 cc. When the milk intake is ample, but the vitamin D intake inadequate, values tend toward the low normal limits. Values for serum phosphorus below 4 mgm. per 100 cc. are considered indicative of active rickets. The organic phosphorus compounds, both of serum and of corpuscles, tend to be lower at birth than during later infancy.

Magnesium. Magnesium, like phosphorus, is utilized both in the skeleton and the soft tissues. The total quantity, however, is small. The magnesium content of the fetus increases to about 200 to 225 mgm. per kilogram by the sixth month of pregnancy and remains at about this

value throughout the remainder of fetal life. Its partition between bone and soft tissue will be discussed in the next section.

Human and cow's milks show the same relative difference in magnesium as in calcium content. Fewer data are available concerning magnesium metabolism during infancy and such data as we have show a wide variation in retention, the values due in part to difficulty of analysis, particularly with the older methods. The amount of magnesium retained from cow's milk feedings is much greater than that from human milk. From 20 to 50 per cent of the magnesium output is excreted in the urine.

Unlike the serum calcium, serum magnesium in infancy appears to be wholly within the adult range of 2 to 3.5 mgm. per 100 cc. No reports were found as to whether the magnesium content of corpuscles is also at the adult level, 5.4 to 7.8 mgm. per 100 cc.

Electrolytes of body fluids. With exception of the minerals associated with special tissues, including the skeleton, the amount of electrolytes in the body will depend on the amount of body water and its distribution between intra- and extra-cellular fluid. The study of composition of body fluids has progressed rapidly in the past few years, particularly through the efforts of the Yale investigators, and the recent findings may be summarized as follows:

Body chloride occurs almost wholly in extra-cellular fluid; the only exception is the chloride of red blood cells, which amounts to 80 m.-eq. per liter of cellular water. About 25 per cent of the total body sodium occurs in an insoluble form in bone and cartilage; the sodium of bone occurs in the proportion of 1 mM of sodium to 30 mM of calcium. The remainder of body sodium is in extra-cellular fluid. Extra-cellular fluid is of constant composition and contains 120 m.-eq. of chloride, 148 m.-eq. of sodium, 5 m.-eq. of potassium, 1 to 2 m.-eq. of magnesium, and 2 to 3 m.-eq. of calcium per liter of water. The cation content of intra-cellular fluid is also constant, each liter of intra-cellular water containing 140 m.-eq. of potassium and 35 m.-eq. of magnesium; the anions—protein, organic phosphate and bicarbonate—vary in amount with the tissue. Muscle tissue has the lowest protein content, containing 300 grams of protein and 100 mM of phosphate per liter of water; liver contains 450 grams protein and 180 mM of phosphate per liter.

Average values for nitrogen, water and electrolyte content of fetuses from values reported in the literature are shown in chart 2. The body content of extra-cellular and intra-cellular water can be readily estimated from the chloride and potassium content. The chloride content

decreases from about 73 m.-eq. per kilogram body weight at the twentieth week of gestation to 50.4 m.-eq. per kilogram at birth. Without correcting for the small amount of chloride in red blood cells, approximately 62.5 per cent of the body weight of the fetus at the twentieth week of gestation is due to extra-cellular water, whereas only 43 per cent of the weight at birth is ascribable to this source.

The potassium content of the fetus increases rapidly to about 38 m.-eq. per kilogram at 24 weeks in utero, then more slowly to the average value of 43 m.-eq. per kilogram at birth. Estimates of extra- and intracellular water from chloride and potassium values (uncorrected) give the following values for fetal water. At 24 weeks the average values are, extra-cellular water 58 per cent and intra-cellular 27.5 per cent; at 32 weeks, 51 and 29 per cent and at birth, 43 and 31 per cent respectively for extra- and intra-cellular water. These values compare very favorably with the average total water content as determined by analysis, 85.5, 79.6 and 72.5 per cent respectively for fetuses at the 24th, 32nd, and 40th week of gestation. The proportion of body weight due to extra-cellular water at birth is still more than double the amount found in the adult, which is 20 per cent of the total body weight. The newborn infant has been likened to the edematous adult in this respect. According to Kerpel-Fronius, throughout life the muscles, skin and nervous system account for 61 per cent of the water content of the body; in the newborn infant, 32 per cent is extra-cellular and 29 per cent intracellular water; while in the adult 20 per cent is extra- and 41 per cent intra-cellular. Whereas in the adult the intra-cellular water of musculature accounts for nearly half the total body water, at birth only 18 per cent of the water content is so found. The skin of the newborn infant contains 21 per cent of the total body water, that of the adult, 9 per cent. Iob and Swanson report that fetal bone is 43 to 46 per cent and cartilage 80 to 85 per cent water. Adult bone contains 10 to 25 per cent water. These variations in water content and distribution cause differences in relative amounts of postassium and magnesium, sodium and chloride present in the body of the newborn infant compared with the adult.

The relative nitrogen content of the body increases more rapidly during fetal life than the rate of increase in intra-cellular fluid. At the 24th week of gestation, the body contains about 1.5 per cent nitrogen, which with an intra-cellular water content equalling 27.5 per cent of body weight, would allow 54 to 55 grams of nitrogen, or about 340 grams of protein per liter of intra-cellular water. By the 32nd week of

intra-uterine life, the body nitrogen has increased to about 1.7 per cent and the intra-cellular water to 29 per cent of body weight, permitting 59 grams of nitrogen (365 grams of protein) per liter of intra-cellular water. At birth the average nitrogen content of 2.01 per cent results in a protein content of 405 grams per liter of intra-cellular water—a value higher than that observed in adults and approaching that of glandular tissue alone. Such a value is in keeping with the knowledge that glandular tissue forms a far larger proportion of body weight in the newborn infant than in the adult, while muscle tissue, which contains a much lower quantity of protein per liter of intra-cellular water, is only 25 per cent of body weight of the newborn infant but about 45 per cent of body weight of the adult.

The magnesium content of the fetus remains almost constant at 18 m.-eq. per kilogram body weight during the last 16 weeks of gestation and is apportioned between intra-cellular water and bone. At 35 m.-eq. per liter of water, the fullterm fetus would use approximately 11 m.-eq. per kilogram body weight in intra-cellular fluids, leaving 7 m.-eq. or 84 mgm. per kilogram for deposition in bone.

To summarize, the chief changes occurring in the last half of fetal life are a steady decrease in the relative amount of extra-cellular fluid, as shown by decreases in the sodium and chloride content per kilogram body weight. The amount of intra-cellular fluid per kilogram body weight increases, but at a much slower rate than the decrease in extra-cellular water, consequently the total water content per kilogram of the fetus decreases. The rate of increase in nitrogenous tissues, as estimated from body content of nitrogen and of intra-cellular water is such as to indicate more rapid growth of glandular than skeletal muscle tissue.

After birth, the changes in relative proportions of body electrolytes as determined by retention studies, would be an efficient tool in aiding to apportion weight gain of infants among various tissues. Reports of retention of these elements by infants must be entirely re-evaluated, however, in the light of the findings of Rominger and Meyer, and of Iob and Swanson that in infants fed cow's milk, from 10 to 15 per cent of the total excretion of sodium, potassium and chloride occurs through the skin. More recently, Levine and his collaborators have observed that the amount of excretion through the skin varies with the temperature and humidity of the surrounding atmosphere. These facts preclude the use of correction factors and make possible only the most general of conclusions concerning comparative retention of infants given different types of feeding.

A comparison of apparent retention (uncorrected for loss through the skin) of sodium and potassium by infants fed human and cow's milk shows that the infants fed cow's milk apparently retain twice as much potassium and about one and one-half times to two times as much sodium as infants given human milk. These differences are so great that even allowing for a high percentage of error, it seems safe to assume that the potassium retention of the infant fed cow's milk is considerably above that of the infant fed human milk. Such an assumption is also consistent with the observed nitrogen retention of the two groups (chart 2). Infants fed human milk do not quite maintain their birth percentage of nitrogen; those fed undiluted cow's milk show a continuous increase in relative body nitrogen at a rate comparable to that predicted according to the constant differential growth ratio. Since the body of the infant fed cow's milk contains a greater percentage of protein than that of the breast-fed infant, the amount of intra-cellular fluid must also be greater and a correspondingly larger retention of potassium, magnesium and phosphorus would be expected.

From the wide difference in uncorrected values for comparative sodium retention, it seems probable that the relative content of extracellular water is also greater in the cow's milk fed infant, though the differences in amount may not be as great as the differences in intracellular water. Human and cow milks are calorically approximately equal, though it is customary to give infants cow's milk feedings of somewhat higher caloric value than would be ingested as human milk. The relative weight gains of the infants fed cow's milk are somewhat greater, a larger percentage of weight gain is due to protein and to water, so presumably a smaller amount is due to fat than in the breast-fed infant. From the constancy with which the curves for nitrogen and calcium follow those estimated by Moulton for body composition on a fat-free basis it appears probable that the fat content of infants fed cow's milk is maintained at approximately the birth level, whereas that of infants fed human milk increases markedly, as does the fat content of suckling young of other mammals.

One complete and well-controlled study of retention of organic foodstuffs, water and minerals is at hand. Gordon, Levine and associates have made use of the fact that the sweat glands do not begin to function until very shortly before birth. Therefore, by using premature infants as subjects, errors due to excretion through the skin were more easily avoided. The infants were kept in an atmosphere of constant humidity and temperature and were closely watched for signs of perspiration.

Concurrent determinations were made of retention of water, organic foodstuffs and electrolytes by four infants. Unfortunately for the purpose of this review no infant was given human milk exclusively so the data provide no evidence for direct comparison of the two types of feeding heretofore considered. The nitrogen and caloric intakes, however, varied from infant to infant. In general, the total water retention averaged about 70 per cent of the total weight gain. The amounts of potassium, and phosphorus retained by the group approximated the amounts estimated from the nitrogen and calcium retention and were consistent with tissue gain of the approximate composition of 54 grams of nitrogen per kilogram of intracellular water. The sodium retention approximated that estimated from the retention of chloride $\left(\text{mM Na} = \frac{148}{120}\ \text{mM Cl}\right)$ and calcium $\left(\text{mM Na} = \frac{\text{mM Ca}}{30}\right)$. Body content of both intra- and extra-cellular water seemed to increase with increased retention of nitrogen. The quantity of fat retained by these infants bore no obvious relation either to caloric or protein intake.

Study of changes in quantity and distribution of body water show that a comparatively wide range both of relative proportion of various tissues of the body and also of relative composition of each must be included as normal.

The electrolyte composition of serum, as of other body fluids, tends to remain constant. The sodium, potassium and chloride content of serum water is the same in infancy as in adult life. Serum at birth has less protein than in later infancy, but the differences are not sufficiently great to make the sodium and potassium content of serum appreciably different from those of other periods of life. The potassium and chloride content of water of erythrocytes is also comparable with that observed in adults. The exact amounts of each present in whole blood will depend on the relative amount of corpuscles present, which is an extremely variable factor in early infancy. Whole blood determinations, therefore, have still less meaning for infants than adults.

Sulfur. The sulfur metabolism of infants has been studied by Blaszó. The sulfur content of human and cow's milk is identical, 0.48 per cent, though the protein sulfur content of cow's milk is larger than that of human milk. The infants retained 82 to 98 per cent of the intake, or 0.053 to 0.076 gram per kilogram daily, whereas the sulfur retention of children 3 to 14 years of age varied from 9 to 59 per cent of the intake, or 0.002 to 0.021 gram per kilogram daily. The amount of

sulfur retained per gram of nitrogen retention is also greater for infants than for older children.

Though very little of the ingested sulfur is excreted, the chief avenue of excretion is the urine. Urinary sulfur of infants is characterized by a higher proportion of neutral sulfur and less inorganic sulfate than the urine of older children and adults.

Iron. The iron content of the fetus under 100 grams' weight is approximately constant at about 0.0050 per cent of body weight. During the rapid growth of late gestation the amount increases to 0.007 to 0.009 per cent. Calculated on dry, fat-free basis, however, the iron content per kilogram tends to be constant throughout fetal life.

At birth, the greatest part of body iron is concentrated in the blood. Though the number of erythrocytes is approximately the same as in adults, the concentration of hemoglobin is usually greater than at any period during later life. The exact amount of hemoglobin varies widely among individual infants, from 15 to 28 grams per 100 cc., but seems to average approximately 22 grams per 100 cc. This high concentration is attributed to the low oxygen tension of the placental circulation. It is stated that a considerable proportion of the hemoglobin is in a form somewhat different from the hemoglobin of adults. After the lungs begin to function, less hemoglobin is needed; destruction of the excess begins almost immediately and is completed by the ninth to twelfth week of life, when the hemoglobin reaches its minimum value of 10 to 12.5 grams per 100 cc. During the decrease in hemoglobin, the amount of nonhemoglobin iron in blood corpuscles increases to about 5 per cent of the total blood iron. After the minimum hemoglobin level is reached, the value increases slowly, attaining 11.5 to 13.5 grams per 100 cc. at about five months of age after which the level remains constant or gradually declines throughout the remainder of the first year. Illness, either gastro-intestinal or infectious, causes a decrease in hemoglobin level. The average values obtained at any age seem to vary with the locality, general standard of health and type of infant dietary, as well as the technique of the investigation. The various factors involved are discussed at length by Faxén.

In addition to the normal high iron content of blood at birth some iron is stored in the liver of the newborn infant. Various studies place the amount of liver iron at from 0 to 100 mgm. with an average value of approximately 50 mgm. The smallest values for liver storage were observed in infants born of anemic mothers. The liver content of iron increases for several weeks after birth. Iron is also stored in the spleen

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but the small size of this organ makes the total amount of much less importance for iron metabolism than that in the blood and liver.

Probably the two most important factors in iron metabolism of infants are the high concentration of hemoglobin in the blood at birth, and the fact that the blood constitutes such a large percentage of the total body weight of the newborn. The blood volume, like the hemoglobin concentration varies widely, but averages about 15 per cent of the total birth weight according to Lucas and Washburn. The proportion of blood to total weight decreases as the infant grows, and is about 10 per cent at a year of age.

The iron content of other tissues increases with the weight of the tissues. Muscle hemoglobin is the largest factor to be considered; throughout infancy muscle remains at a constant proportion of about 25 per cent of the body weight and contains about 5 mgm. of iron per kilogram. The average amount of body iron at birth is such that if no iron were lost from the body, none need be retained by the infant from the food ingested during the first six months of life, in order to maintain a blood hemoglobin of about 12 grams per 100 cc. Between six months and 1 year of age, about 0.7 mgm. would have to be retained daily in order to maintain the given concentration of hemoglobin.

The hemoglobin level in the blood of premature infants is approximately the same as for full-term infants. Because of their smaller size, the total amount of hemoglobin is much less than for the average normal infant. The period of physiologic blood destruction lasts as long as that of full-term infants and the amount of decrease in hemoglobin may be somewhat exaggerated. Even though no iron were lost from the body during the period of blood destruction, the rapid growth of the infant and the lowered amount of iron at birth would result in a shortage of body iron, and the development of anemia especially after six months of age.

Infants born of anemic mothers often show hemoglobin values within normal limits, though values as low as 13 grams per 100 cc. are reported. These infants seem rarely to have additional stores of iron in the liver and commonly become anemic during later infancy.

Studies of infants under three months of age show that both breast and artificially fed infants tend to lose small amounts of iron during the period of the hemoglobin breakdown, a fact neither unexpected nor alarming. The non-hemoglobin iron of blood at this age is greater than normal, which according to McCance and Widdowson leads to excretion of iron unless the intake is very high. However, the iron intake of the

average baby is meager. The iron content of milk is very low and, especially for human milk, very variable, values from 0.45 to 1.5 mgm. per liter being recorded. According to Wallgren, the higher values are found during early lactation. The iron content of cow's milk as secreted is given as from 0.15 to 0.6 mgm. per liter. Market milk, however, may have an increased content, depending on the length of time the milk is in contact with iron containers.

Feeding additional iron during early infancy apparently does not result in retention unless the amounts given are large; even then retention is only temporary. Opinions as to the effect of iron feeding on the rate and duration of blood destruction vary; evidence has been presented that prophylactic feeding of small amounts of iron shortens the period of physiological hemoglobin breakdown in premature infants and permits a level equal to that maintained by healthy full-term infants.

After three months of age, iron retention is variable, but in general it may be stated that infants fed human milk usually retain very small amounts of iron. Retentions recorded by Wallgren vary from −0.14 to +0.5 mgm. daily, with an average retention for four infants of 0.11 mgm. a day. Infants above three months of age fed cow's milk may gain or lose iron, but the amounts involved are negligible whereas a retention of at least 0.7 mgm. is needed daily to maintain hemoglobin level after six months of age. Thus, when the infant is fed only the milk formula, the body is poorer in iron by six months of age than at birth, and becomes progressively poorer with continued growth. The development of nutritional anemia during the last six months of infancy is almost inevitable, therefore, unless iron is given in addition to that supplied from the milk formula. If in addition to the low intake, infection or illness causes loss of iron from the body, the development of anemia is hastened.

It has become customary to feed supplementary foods much earlier in infancy than was formerly the case. Of these foods the two most often used before five months of age are egg yolk and cereal. An egg yolk adds 0.5 to 1.0 mgm. to the day's intake of iron; the iron of cereals varies from 0.2 mgm. per ounce for highly refined cereals to 17.4 mgm. per ounce for those fortified with iron. Vegetables and fruit purees contribute from 0.1 to 0.5 mgm. per ounce. Infants seem to utilize the iron of meat, which is largely organic, as well as inorganic forms of iron; the iron of oxalate-containing leafy vegetables, however, seems not to be utilized by infants. Soluble iron salts, both ferrous and ferric, are well utilized by infants. Iron excretion is almost wholly by way of the

feces. From retention studies, the iron requirement of artificially fed infants over three months old has been placed at greater than 0.5 mgm. per kilogram and close to 1 mgm. per kilogram daily. On the basis of blood studies only, an intake of 25 mgm. of iron and 1 mgm. of copper daily is stated to be necessary for the maintenance of optimal hemoglobin levels. It is difficult to maintain an intake of 1 mgm. per kilogram from food alone; it would be impossible for infants to attain an intake of 25 mgm. of iron daily without the use of iron salts. The actual iron requirement in infancy varies with several factors, not all of which are as yet understood. The low gastric acidity of infants is probably not an important factor except that the anacidity occurring during infection probably contributes to the impaired utilization of iron at that time. The thiamin intake may be a factor. Adequate intakes of vitamin C and of copper are known to be essential for utilization of the retained iron, vitamin C for proper functioning of bone marrow and copper for the formation of hemoglobin.

Copper. The copper content of fetal blood at 20 to 25 weeks' gestation is reported as 0.195 to 0.235 mgm. per 100 cc.; that of full-term infants from 0.073 to 0.082 mgm. per 100 cc. Average values reported for older infants vary from 0.100 to 0.176 mgm. per 100 cc. Lesné and Briskas report that blood levels of iron and copper are reciprocal, and the blood copper of anemic infants is above normal. Copper is stored in the liver at birth, and the amount decreases during infancy.

The copper content of human milk is reported as from 0.21 to 0.28 mgm. per liter; that of cow's milk from 0.09 to 0.21 mgm. per liter. Pasteurization or evaporation in copper tanks increases the copper content of milk. The supplementary foods used in infancy also contain small amounts of copper. The copper requirement of the infant is not known, but it has been definitely established that hemoglobin regeneration is more rapid when anemic infants are given copper as well as iron in amounts greater than those provided by the diet. The ratio of 1 mgm. of copper to 25 mgm. of iron is stated to be most effective in increasing the hemoglobin content of blood. If the iron requirement is placed at 1 mgm. per kilogram body weight, the copper intake should be met by an intake of 40 micrograms per kilogram. Such an intake cannot be met from cow's milk alone.

Cobalt. Cobalt sulfate in doses of 25 to 50 mgm. daily together with 0.5 gram of iron a day is reported to cause an average gain in hemoglobin of 0.75 gram per 100 cc. weekly for 3 to 4 weeks in mildly anemic infants after treatment with iron alone has failed to cause hemoglobin increase. No toxic symptoms were noted during the progress of the study. The

amounts of both substances given seem excessive and the copper content of the salts was not determined. In view of the very small amounts of cobalt needed for cure of anemia in animals and the fact that the amounts reported to produce polycythemia in animals are not large, it seems that the use of cobalt salts for anemia of infants should be attended with caution. Recent studies indicate that cobalt is probably not necessary for normal hemoglobin metabolism in human infants fed milk.

Iodine. The knowledge of iodine metabolism during infancy is still far too scanty. Iodine deficiency in the mother results in hypothyroidism in the infant. In regions where goiter is prevalent the use of iodized salt (1:5000) throughout pregnancy is advocated for normal women as an effective means of preventing thyroid deficiency in herself and her infant. In the Swiss Canton of Bern, where iodized salt contains only 5 mgm. of iodine per kilogram but other forms of iodine prophylaxis are common, the average weight of thryoid in the newborn has decreased from 10 to 6.6 grams in the last thirty years. Scammon states that the weight of thyroid in the normal newborn infant is 1.5 to 2.5 grams and larger thyroids are evidence of iodine deficiency. It appears that the higher iodine content of American iodized salt is a better safeguard than the smaller amount in Swiss salt. Crile and Crile advocate a still larger prophylactic dosage, 10 mgm. of iodine weekly for all non-goiterous pregnant women in regions where goiter is prevalent.

The iodine content of the mother's blood increases during the latter half of pregnancy to from 20 to 40 micrograms per 100 cc., about double the level in non-pregnant women. The iodine content of the cord blood is from 10 to 20 micrograms with an average of about 15 micrograms per 100 cc. In later infancy the average blood iodine decreases to between 5 and 10 micrograms per 100 cc.

As would be expected, the thyroid contains the largest percentage of body iodine at birth. The amount of iodine increases both relatively and absolutely with the size of the gland. According to Lelkes, in normal fetuses, the total iodine content of the thyroid is rarely over 10 micrograms, but in enlarged thyroids the iodine content is increased. Palmer and associates found the iodine content of the thyroid of newborn infants averaged 49 micrograms, 20 per cent of which occurred as thyroxin. The amounts of iodine found by Palmer in the thyroids of newborn infants equalled the amounts observed by Lelkes in the thyroids of older infants, values of 48 to 168 micrograms being reported. The thyroids examined weighed 1.5 to 3.0 grams.

The iodine content of both human and cow's milk is variable, depend-

ing on the iodine content of the food. The percentage amount in colostrum is greater than that of milk, varying from 12 to 45 micrograms per 100 cc. for human colostrum and from 13 to 17 micrograms per 100 cc. for cow's milk. The iodine content of human milk after lactation is established varies from 2 to 15 micrograms per 100 cc.; for cow's milk, iodine values of 0.3 to 187 micrograms per 100 cc. have been reported, the high value being from milk of cattle near the seacoast. The iodine content of cow's milk has been increased about 10-fold by feeding iodine. The iodine of milk is associated with the milk fat. If skimmed milk is fed, or if milk fat is replaced by other fat of lower iodine content the iodine content of the milk feeding will be markedly reduced.

The increased blood iodine at birth and the relatively high iodine content of colostrum are often considered as means of increasing the iodine storage of infants. It is worthy of comment, however, that the actual day's intake of iodine is greater after lactation is established than when colostrum is given, due to the difference in quantity of intake.

Of the other foods in the infant dietary, cod liver oil is one of the best sources of iodine. American cod liver oil contains 3.6 to 15 micrograms of iodine per gram. A teaspoonful daily (3.5 grams) thus adds 12 to 50 micrograms of this element to the day's intake. The iodine content of the other articles of the baby's diet varies even more widely than the iodine content of milk. The amount of iodine in eggs varies according to the iodine intake of the hen. Of foods in general, green vegetables, milk and fruit contain the largest amounts of iodine, cereals much less; the exact amounts present vary with the soil content.

The iodine intake of the young breast-fed infant may vary from about 25 micrograms to as much as 140 micrograms daily, the latter if the milk iodine is high and cod liver oil is given; that of the older infant given cow's milk may be anywhere from 3 to 600 or more micrograms daily.

In view of the variability of the iodine content of human milk and its susceptibility to change with the iodine intake of the mother, the amount of iodine in reported samples of human milk cannot be accepted as a guide to the iodine needs of the infant. The fact that intakes varying from 3 to 600 or more micrograms daily apparently do not result in demonstrable ill effects shows that excess is either quickly excreted or stored, or that with iodine as with many of the other elements considered, the range of body content which must be considered normal is very wide.

In this respect, the findings of Remington and Remington may be of significance, that 50 or 100 times the minimal protective dose for iodine did not affect growth or utilization of food for growth in growing rats. Knowledge of optimal iodine requirement in infancy must await further investigation.

Other minerals. Little if anything is known concerning the manganese, zinc or fluorine requirements of infants. The amounts of these substances reported in cow's milk are as follows: manganese, 10 to 40 micrograms, zinc, 3.4 mgm. (average), and fluorine, 0.05 to 0.25 mgm. to the liter. In districts where the fluoride content of water is above 0.8 parts per million, infants should be specially protected. Apparently the tolerance for fluorine in food is considerably greater than for fluorine in water.

In conclusion, it may be stated that neither the minimal nor the optimal requirements of the infant are definitely known. Study of the mineral metabolism indicates that a surprisingly wide range of body composition seems to be compatible with health during the period of infancy.

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